Regulation of Programmed Cell Death in the Developing Thalamus

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Disclaimer

All experiments presented in this thesis were performed by me unless otherwise indicated below.

1) The *in vitro* experiments in Chapter 3 were done in collaboration with Beau Lotto.

2) Most PCR and BrdU immonoreactions were performed by Katy Gillies.

3) Many histological processes were with the great help of Grace Grant and Vivian Alison.

No part of this work has previously been or is being accepted for any other degree.

Pundit Asavaritikrai

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Table of Content

Abstract

Regulation of programmed cell death in the developing thalamus

Programmed cell death (PCD) plays a fundamental role during development of the vertebrate nervous system. At the time of target innervation, many neurons within specific neuronal populations die (most, if not all, by apoptosis) and neuronal survival appears to be controlled by target-derived survival promoting (trophic) support. In many cases, the trophic factors are members of the neurotrophin family. In thalamus, the occurrence and the regulation of PCD are still unclear. In this thesis, I examined PCD in the thalamus during the period of innervation of the cortex (late embryonic to early postnatal ages in mice). I found that apoptosis (revealed by TUNEL and pyknotic morphology) is a common mode of thalamic PCD both *in vivo* and *in vitro*. *In vivo* studies showed the highest rate of cell death is in early postnatal life, at postnatal day 1 (P1). *In vivo* analysis of animals lacking functional neurotrophin tyrosine kinase receptors, TrkB and TrkC, and *in vitro* work in collaboration with Dr R.B. Lotto showed that brain-derived neurotrophic factor (BDNF), acting via TrkB, regulates thalamic survival in the perinatal period. My *in vitro* studies showed that thalamic cells cultured from E15 for up to 5 days showed a loss of viability after 2-3 days, which precedes the *in vivo* increase in thalamic PCD. Cortical factors in addition to BDNF are able to maintain thalamic viability for longer periods in culture. Later studies showed that these control factors are not unique to the cerebral cortex but can be found in other neuronal tissues. Amongst other tissues, the late-gestation thalamus is able to produce them provided it is stimulated with elevated levels of K^+. Elevated K^+ is known to promote thalamic survival by increasing depolarisation but I found evidence that elevated K^+ did not require TrkB or TrkC signaling to produce a trophic effect since K^+ had normal trophic effects on the thalamic explants of either *trkB-/-* or *trkC-/-* animals. Moreover, I observed an increased cell death in the E19 thalamus in mice homozygous for a mutation of the transcription factor *pax-6*. This mutant is known to lack thalamocortical innervation and my *in vivo* and *in vitro* analyses of this mutant suggested that thalamocortical innervation is essential for developing thalamic cells

to obtain sufficient trophic factors. Finally, I observed that the neurotrophins might play not only a survival role but also a proliferative role, as Bromodeoxyuridine (BrdU) studies of mice lacking TrkC signaling showed proliferative defects in the occipital cortex.

Chapter 1: General Introduction

The development of the vertebrate nervous system, like the development of all other biological systems, consists of both progressive and regressive phenomena. The progressive phenomena include induction, proliferation, migration, differentiation, and the development of functional contacts and cellular communication. Because of their essentially additive character, the progressive phenomena had dominated scientists' concepts of the nervous system's development and often overshadowed the significance of the regressive events during development.

The regressive features had not been regarded as playing a prominent role in ontogeny (or some researchers even had totally denied their existence) until the late 1940's. That decade was when Levi-Montalcini and Hamburger found evidence of naturally occurring cell death and cell death following target ablation (limb bud removal) in developing sensory ganglia and motor nuclei (Levi-Montalcini and Hamburger, 1949). Not long after, other forms of regressive events were also discovered. These include axon retraction and synaptic elimination (reviewed by Oppenheim, 1991).

Increasing evidence from subsequent studies in morphology, biochemistry, and genetics confirmed the occurrence of these natural regressive phenomena (Oppenheim, 1999; Clarke, 1990; Clarke, 1994; Pettman and Henderson, 1998). A substantial amount of cell death is found particularly in the vertebrate nervous system. As much as 50% or more of cells in many neuronal populations die during development (Raff et al., 1993). In terms of evolutionary processes, it was thought that such massive death should offer some advantages to the organism. This death must have some specific purpose or serve critical functions for appropriate development, possibly in cell selection, control of cell number, or biological sculpturing in embryogenesis. Thus, the study of naturally occurring cell death becomes a critical and fundamental issue in development.

Terms and Types of Cell Death during Development

Although it was first discovered and extensively studied in the nervous system, cell death during ontogeny also takes place in many different types of tissues. Several terms that are used to classify cell death in general can apply to cell death in the nervous system. But some of these classifications may involve other types of cell death in addition to the death that occurs in development.

Generally, cell death can be classified into two broad categories – pathological cell death and physiological cell death (Kerr et al., 1972; Duvall and Wyllie, 1986). Pathological cell death results from accidents, injuries, and all disease-related conditions. In contrast, physiological cell death occurs in normal conditions, and includes cell turnover in homeostasis and normal cell death during development. Various terms are used to describe the latter, such as normal cell death, spontaneous cell death (Oppenheim and Chu-wang, 1977), naturally occurring cell death (Ferrer et al., 1992), developmental cell death (Henderson, 1997), or programmed cell death (PCD) (Oppenheim, 1991). The meanings of these terms are very similar and sometimes interchangeable. The specificity of the terms used might vary slightly. For instance, developmental cell death can mean any kind of cell death occurring in development, which includes cell death by normal homeostatic mechanisms taking place in ontogeny.

Only the term 'programmed cell death' or PCD has the clearest definition for the specific death that occurs during development. It is described as "*a spatiotemporally-reproducible and specific loss of large numbers of individual cells which occurs only in time of development*" (Oppenheim, 1999). This excludes a proportion of cells that might also die during development but that does not serve any distinct functions related to embryogenesis, or that dies due to normal homeostasis. By this original definition PCD itself does not imply any particular mode of cell death, classified by morphology and/or biochemical processes, although later studies might find that a certain mode of morphologically classified death is more characteristic to PCD than others (Oppenheim, 1999).

Based on morphology alone, cell death during development can be classified into at least three types: apoptotic (type I), autophagic (type II), and cytoplasmic (type III) (Clarke, 1990). However this 'threefold' classification is not popular for several reasons. 1) There may be overlapping features among these three types in some dying cells. For instance, type II cell death (which is characterised by autophagic vacuoles) may exhibit pyknosis (resembling type I), and cytoplasmic dilation (as type III). This classification is thus difficult to interpret. 2) The classification lacks supporting biochemical evidence to distinguish each type from others (Clarke, 1990).

A similar but more general cell death classification has been used in both pathology and physiology. This is based on both biochemical processes and morphological appearance. Two broad terms are used to describe cell death in this classification – necrosis and apoptosis (Wyllie, 1998).

Necrosis is morphologically similar to the type III cytoplasmic cell death that occurs during development. However, necrosis is involved primarily in pathological conditions and can be regarded as 'cell murder'. It is death caused by external damage, usually involving destruction of cell integrity (Schwartz et al., 1995; Schwartz and Osborne, 1995). The necrotic cell exhibits a swollen cytoplasm, its plasma membrane lyses and releases debris which often induces inflammation. In essence, this event happens as a passive response to injury. Biochemically, the cell loses its regulation of ionic homeostasis. Many lysosomal enzymes are released and activated. DNA is degraded with non-specific patterns. Necrosis can occur in a matter of seconds (Collins et al., 1997) but the subsequent repair and scarring might take a much longer time or even be permanent throughout the life of an organism.

Unlike necrosis, apoptosis is characterised by nuclear and cytoplasmic condensation and the disintegration of the cell into a number of well preserved, membrane-bound fragments (Kerr et al., 1972). These appearances result from the activation of many reactions of the cell – intrinsic 'cell suicide' machinery. This does

Figure 1.1

Apoptosis and necrosis: (I) A healthy cell is depicted undergoing stages of apoptosis (1-5) or necrosis (1'- swelling, and 2'- lysis). In apoptosis, cell-membrane-bound apoptotic bodies (2) are engulfed and degraded by a macrophage (3-5). (II) Apoptotic and (III) necrotic motoneurons as seen in the electron microscope. (IV) DNA extracts on gel electrophoresis; note a DNA fragment laddering in apoptotic cells (Oppenheim, 1999).

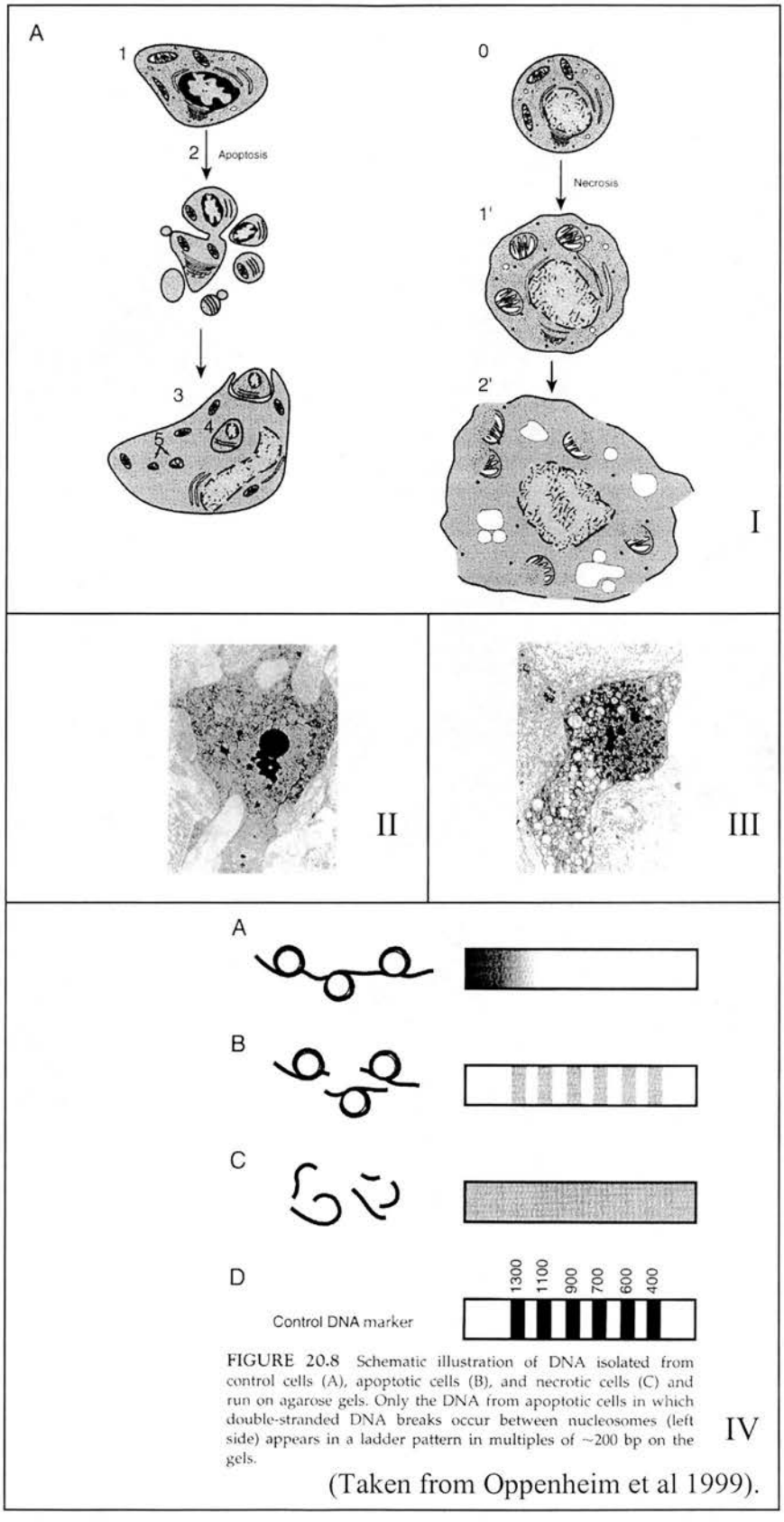


FIGURE 20.8 Schematic illustration of DNA isolated from control cells (A), apoptotic cells (B), and necrotic cells (C) and run on agarose gels. Only the DNA from apoptotic cells in which double-stranded DNA breaks occur between nucleosomes (left side) appears in a ladder pattern in multiples of ~200 bp on the gels.

(Taken from Oppenheim et al 1999).

not appear to involve lysosomal enzymes. Biochemical detail is discussed in the next section. The processes take a much longer time from the induction step than those of necrosis. However, once the cellular machinery is committed to death, the degenerative process (including elimination) is rapid with cells dying and being removed in minutes or a few hours. The prepackaged membrane-bound components are engulfed by macrophages, microglia, or even by their neighboring cells (Kerr et al., 1972).

Apoptosis is not a mechanism restricted only to development, and it is not a synonym of physiological cell death as it was originally thought. It can be found in many pathological conditions. In non-developmental physiological states, apoptosis controls the rate of cellular turnover in adults (a homeostatic mechanism to balance cell proliferation). Certain hormones, such as glucocorticoids, also induce apoptosis in both physiological (normal) and pathological (neoplastic) cytotoxic T-lymphocytes (Wyllie, 1980). Other examples of apoptosis in pathology include apoptosis following cellular repair, following injury, and in certain neurodegenerative diseases (Kerr et al., 1972; Isacson, 1993)

Some of the cell death that occurs during normal development may not involve the mechanisms of apoptosis (reviewed by Sanders, 1997) and, by definition, apoptosis is not synonymous with PCD. Nevertheless, in many cases the observation of developmental apoptosis implies that PCD is occurring. They both appear to serve certain distinct functions related to embryogenesis. PCD exhibits specific morphology (such as pyknosis) and has the cytochemical properties of apoptosis. At least one apoptosis-regulating gene, *bcl-2*, also controls PCD (Oppenheim, 1999). The highest level of *bcl-2* expression is found in neurons during the period of PCD, which is also the time of target innervation (Holm and Isacson, 1999). Most studies of PCD rely on detection of apoptosis. Furthermore, the underlying mechanisms in apoptosis are relatively well understood. For these reasons, in practical use, apoptosis during development can represent PCD. In development, we can refer to apoptosis as PCD.

Molecular Mechanism of Apoptosis

Apoptosis consumes energy. At the DNA level, a key feature of apoptosis is chromosomal DNA fragmentation, which uses energy. DNA digestion produces small, double-stranded fragments of approximately 185 base pairs that migrate along electrophoretic agarose gels in multiples and generate a characteristic 'DNA ladder' pattern (Arends et al., 1990). DNA fragmentation in apoptosis, unlike that in other non-apoptotic processes, selectively creates 3' overhang DNA fragments in a much higher proportion than the other two types of fragments (5'overhang and blunt end DNA) (Didenko and Hornsby, 1996). The 3'overhangs are also preferred substrates for terminal deoxynucleotidyl transferase (TdT) which is used widely for an apoptosis detection method, the *in situ* DNA fragment labeling technique named TUNEL (*T*dT-mediated *dU*TP-biotin *n*ick *e*nd *l*abeling) (Gavrieli et al., 1992).

Apoptosis can be genetically predetermined, or regulated by epigenetic control (see review in Cowan et al., 1984). It is triggered via either intrinsic or extrinsic factors (Figure 1.2). Intrinsically, certain transcription factor genes such as *ces-1* (*C. elegans* cell death specification) and *ces-2* determine cell fate. Activation of these genes in specific groups of cells leads to cell death. Some metabolic perturbation such as an altered cellular oxidation-reduction process may result in release of many apoptotic mediators (Green and Reed, 1998). Failures to repair damaged DNA (e.g. following UV irradiation, Wyllie, 1980) also directly triggers apoptosis without acting via membrane receptors.

Extracellular signaling can induce cell death in two ways: 1) by triggering death receptors and 2) by trophic deprivation or down-regulation of survival (trophic) receptors. The first was initially discovered in the immune system. Apoptosis is triggered by transmembrane death receptors of the tumor necrosis factor receptor (TNFR, or Fas/TNFR) family. The detail of the pathway is not well understood, but it has been thought to involve the sphingomyelinase pathway and Ca^{++} release. The analogous death receptor in the nervous system is the p75 neurotrophic receptor, which is also a member of Fas/TNFR family (Pettman and Henderson, 1998).

Figure 1.2

Summary of the events occurring in apoptosis: Triggering of apoptosis by external or internal factors proceeds in 4 steps; activation, propagation, commitment, and death. The first two processes are presented in more detail on neurotrophin signaling in figure 1.3, and the latter two are diagrammed in Figure 1.4.

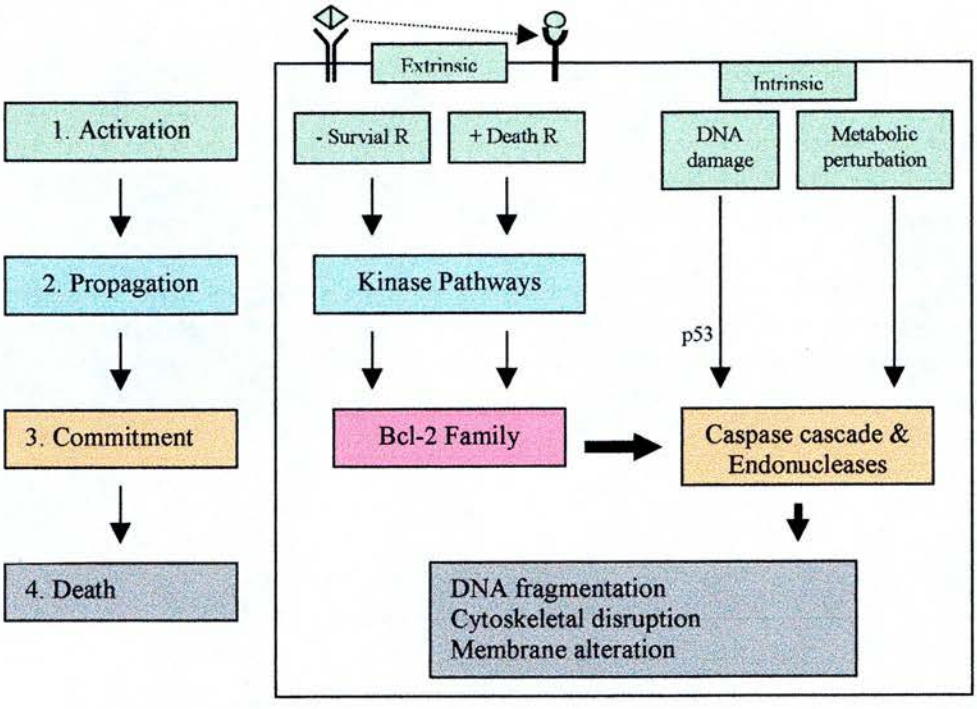

1. Activation
2. Propagation
3. Commitment
4. Death
Extrinsic
Intrinsic
- Survial R
+ Death R
DNA damage
Metabolic perturbation
Kinase Pathways
p53
Bcl-2 Family
Caspase cascade & Endonucleases
DNA fragmentation
Cytoskeletal disruption
Membrane alteration

Figure 1.3

Neurotrophins, binding receptors and their signal transduction

A) Neurotrophin receptor binding: NGF binds TrkA (sometimes called Trk), BDNF and NT-4 bind to full-length (functional tyrosine kinase) and truncated (non-catalytic) TrkB. NT-3 binds to all receptors with a preference for TrkCs.

B) Neurotrophin signal transduction: Signals through Trk receptor are divided into 2 major pathways. Ras dependent pathway induces the activation of Raf protein and the phospholipase C (PLC-γ) pathway. Both can increase cellular metabolism, via the action of MAPKK (mitogen activation protein kinase-kinase) and protein kinase C (PKC, in association with calcium). 2) Ras independent pathway regulates cell differentiation (the function prominently found for NT-3) via SNT protein, and prevents cell death by PI-3 kinase (PI3-K).

P75 receptor, on the other hand, acts via IkB and sphingomyelinase to promote apoptosis. The detailed mechanism is still unclear. However, at the same time, it is the same p75 receptor that helps increase Trk affinity binding to neurotrophins. The contradictory functions are not fully understood.

Abbreviations: TRK = tyrosine kinase receptor (including all neurotrophin receptors), TNFR = tumor necrosis factor receptor, Ras (in)dep. P'w = Ras (in)dependent pathway, -K = kinase, MAPK = Mitogen activating protein kinase, TFs = transcription factors, ieg = immediate early genes, drg = delayed responsive genes, PLC-γ = phospholipase C gamma, DG = Diacylglecerol, PIP2 = Phosphatidyl inositol 4,5 bisphosphate, IP3 = Inositol 1,4,5 trisphosphate. PKB = Protein kinase B, PKC = Protein kinase C, Ca^{++} = Calcium ion, CREB = cAMP responsive element binding protein. Other molecules are known by their full names.

Neurotrophin Signaling, (modified from Segal and Greenberg, 1996)

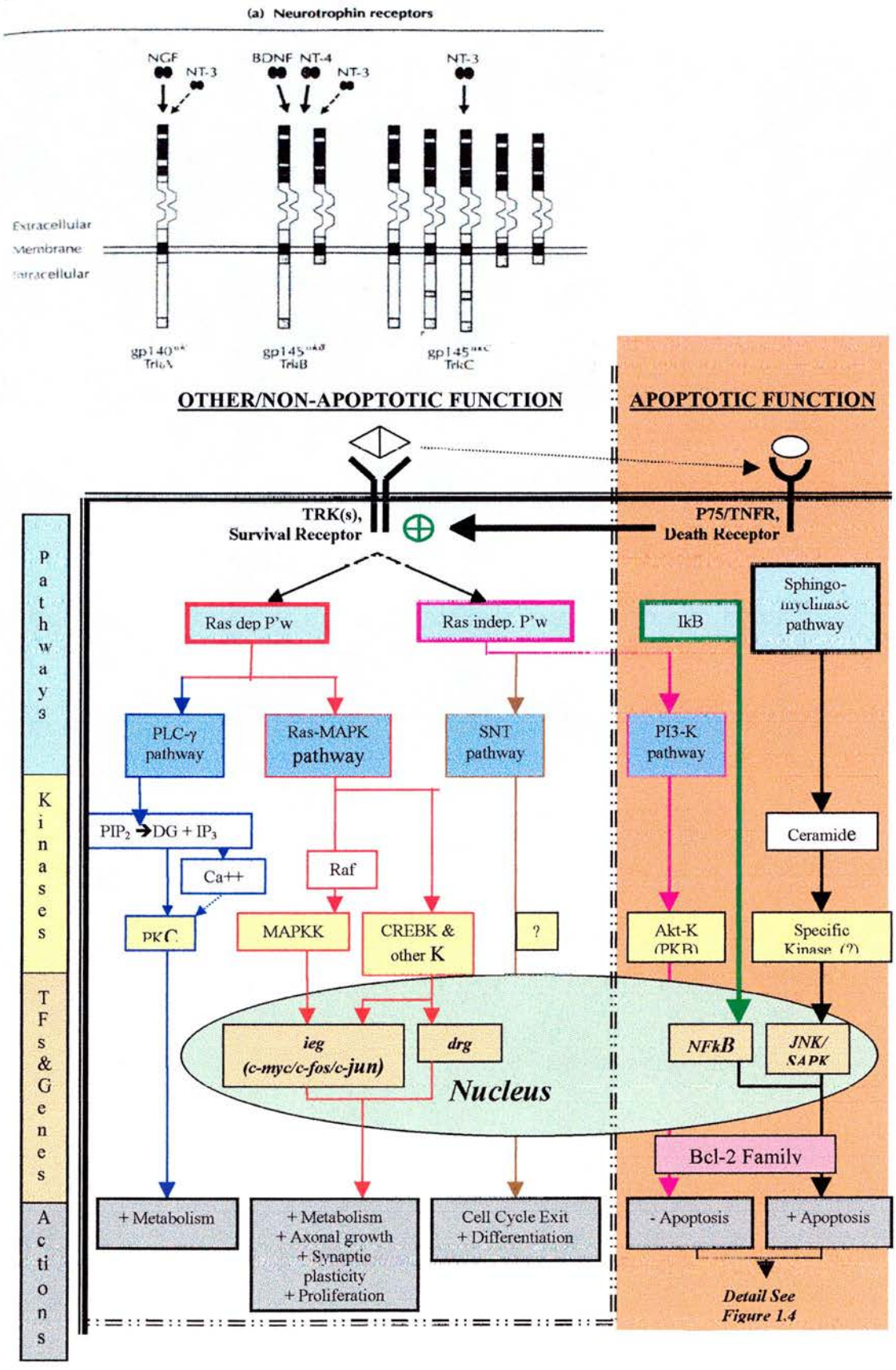


Figure 1.4

Survival and death receptors in caspase dependent apoptosis

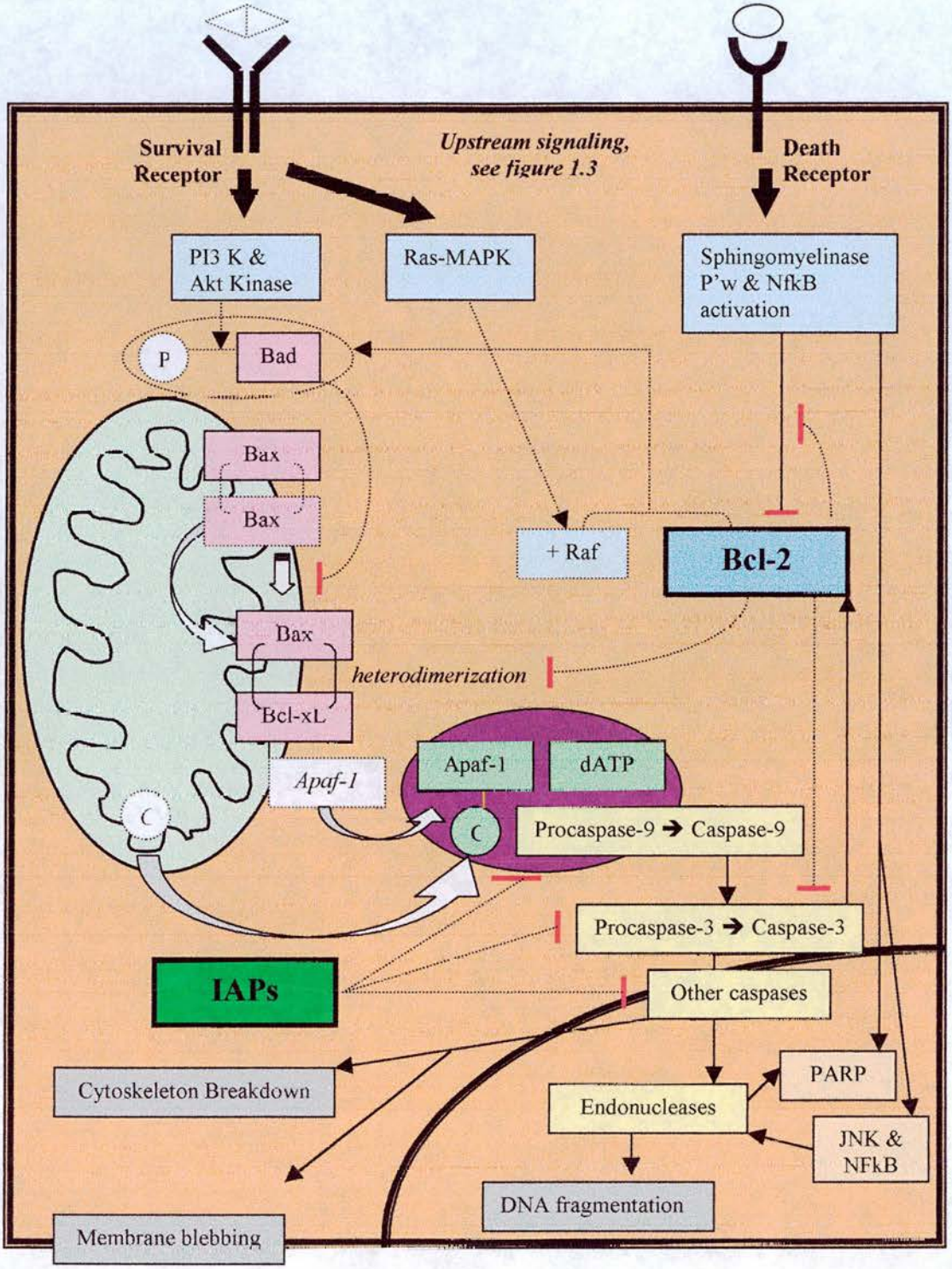
The Bcl-2 family plays a major role in the caspase dependent pathway for the induction of apoptosis. One member of this family, Bax protein, which normally is in dimer form, would heterodimerise with Bcl-xL if Bad is dephosphorylated. Once heterodimerised, Apoptosis protease-activating factor (Apaf-1) and Cytochrome C (©) will be released from the mitochondria form a complex with dATP and procaspase-9 which then activates itself and then activates a key enzyme for apoptotic degradation, caspase-3. This results in DNA fragmentation, cytoskeletal disruption, and membrane blebbling.

The mechanism of apoptosis signaling through the Death receptor is not clear but is thought to involve the Sphingomyelinase pathway, which can i) inhibit Bcl-2 (see below) and ii) activate NFkB transcription factor. The latter can increase several caspases, endonucleases, and also polyadenosine dinucleotide-ribose polymerase (PARP) (the enzyme known to involve in DNA fragmentation).

Activation of the survival receptors can keep the cell alive by keeping Bad phosphorylated via I) PI3 K/Akt kinase, and ii) Increasing Raf products which, together with Bcl-2, increases the phosphorylated Bad and inhibit apoptosis. Prevention and inhibition of apoptosis can mediate via Bcl-2 or the Inhibitors of Apoptosis (IAP) family of protein. The former helps increasing the availability of phosphorylated Bad, inhibits Bax/Bcl-xL dimerisation, and directly inhibits caspase-3. But when the trophic factors are deprived, these functions (dotted lines) are stopped. IAPs inhibit apoptosis by preventing proteolytic processing of procaspases.

Dotted lines/boxes show mechanisms which normally inhibits of apoptosis, but they will be inactivated if the trophic factors are deprived. Lines with arrows show activation mechanisms whereas the lines with red-line ends represent the inhibition processes. Curved arrows mean the release of mitochondrial molecules to cytosol.

Caspase dependent pathway induced by death receptor or trophic deprivation (modified from Pettman and Henderson, 1998)



The induction of apoptosis by deprivation of target-derived trophic factor was originally described as a regulator of PCD in the nervous system. Certain populations of neurons require some essential molecules for their survival during the period of their dependency on other cells to which they project (their targets). Neurotrophic factors and the neurotrophin family in particular are such molecules, being essential for the activation of survival receptors to keep cells in a number of neuronal classes alive. Deprivation of trophic factors leads to molecular consequences causing apoptosis. This process might directly involve protease cascade pathways. Details of the signaling pathways of the neurotrophins are discussed later on in this chapter.

Much understanding of the molecular mechanisms of apoptosis came initially from studies in the invertebrate, *Caenorhabditis elegans*. The cell death pathway in this animal is in many ways a miniature version of the vertebrate apoptosis pathway. Several genes responsible for cell death in this nematode have been characterised and are called *ced* genes (*C. elegans* death genes) (Hengartner, 1998). Key genes regulating apoptosis include a negative regulator (ced-9) and two positive regulators (ced-4 and ced-3). Proteins encoded from each of these genes have their functional equivalent in mammals: the negative regulator CED-9 is a member of the **Bcl-2 family** of apoptosis regulators, CED-4 has a functional equivalent in apoptosis protease-activating factor-1 (**Apaf-1**), and CED-3 is the prototypical **caspase** (see review in Hengartner, 1998; Pettman and Henderson, 1998).

A major biochemical pathway for the execution of cell death programmes is cascade activation of the cystein protease family. These cystein proteases are called caspases (caspase 1-13 have been identified in mammal) (Pettman and Henderson, 1998). Not all caspases are thought to be involved in apoptosis but there are at least two key enzymes, caspase-3 and caspase-9, known for their roles in regulating neuronal apoptosis. In particular, caspase-3 activation plays a central role in apoptosis of many neuronal classes (Kuida et al., 1996; Du et al., 1997). This process requires four components; activation of pro-caspase-9, Apaf-1, cytochrome C (cytosolic), and dATP.

When the level of Bcl-2 (an anti-apoptotic gene; see below) is high, Apaf-1 and cytochrome C normally reside in the mitochondria. But when the level of Bcl-2 is low or there is an increase in free cytosolic Bax (a pro-apoptotic gene; see below), Apaf-1 and cytochrome C are released from mitochondria and form a complex with cytosolic pro-caspase-9 and dATP. When the complex is formed, the active caspase-9 activates caspase-3 which leads to morphological changes in the cell including DNA fragmentation, membrane and cytoskeletal alterations. DNA fragmentation is the result of one key endonuclease activated by caspase-3, caspase-activated DNase (CAD) (Enari et al., 1998). However, it should be emphasised here that, this DNA cleavage alone does not necessarily determine cell death; instead, the death of a cell is the loss of the ability to maintain its intracellular homeostasis. Inhibition of DNA cleavage by mutation of CAD does not prevent cell death (Sakahira et al., 1998). Another consequence of cell death is also destabilisation of membrane, exposing phosphatidyl serine to the extracellular side of the membrane. Phosphatidyl serine can bind to annexinV and can be used for another apoptosis detection technique. The activation of these caspases is strictly regulated by a family of protein called Inhibitors of Apoptosis (IAPs). These proteins contain a BIR (baculovirus IAP repeat) domain near the amino-terminus that can bind several caspases. Many of the proteins block proteolytic activation of caspase-3, caspase-7 and/or block cytochrome c-induced activation oof caspase-9, thereby preventing initiation of the caspase cascade (Deveraux and Reed, 1999) (Figure 1.4).

Other important apoptotic regulators include the Bcl-2 family members, Bcl-2, Bcl-x, Bax, and Bad. All play a critical role in the regulation of apoptosis (Pinon et al., 1997). Bcl-2 has an anti-apoptotic activity via many active mechanisms. 1) With Raf protein (Figure1.3), it inhibits the release of cytochrome C from mitochondria to cytosol. 2) It prevents the generation of reactive oxygen species in the mitochondria. 3) It can also directly inhibit caspase-3. 4) Lastly, it inhibits ceramide formation, the product of p75 neurotrophin receptor (Greenlund et al., 1995).

Bcl-xL is normally bound to the Apaf-1. In living cells, Bax is normally a dimer. A nonphosphorylated Bad cleaves Bax dimers to Bax monomers. The Bax monomers heterodimerise with Bcl-xL monomers and cause a release of Apaf-1 when apoptosis is triggered. The released Apaf-1 and cytosolic cytochrome C, with ATP, activates caspase-9. In brief, Bcl-2, Bcl-xL, and phosphorylated Bad have anti-apoptotic activity while free Bax, phophorylated Bad, and heterodimer Bax/Bcl-xL are proapoptotic. Summarised events are shown in Figure 1.4.

In the nervous system, many of these apoptosis regulating proteins (Apaf-1, caspases, IAPs and Bcl-2 families) have been shown to regulate the neuronal PCD by genetic manipulation studies. Overexpression of *bcl-2* caused an enlarged brain, increases neurite outgrowth, protects PCD in both CNS and PNS (Holm and Isacson, 1999). However, in Bcl-2 deficient mice, the number of most of subpopulations of neuron appeared to be normal (Michealidis et al., 1996). Targeted disruption of apaf-1, caspase-9, or casepase-3 lead to decreased neuronal apoptosis in the embryonic nervous system and severe gross structural abnormalities (Roth et al., 2000; Yoshida et al., 1998; Cecconi et al., 1998; Kuida et al., 1996; 1998; Hakem et al., 1998).

It was thought that the caspase cascade is a universal and central pathway in apoptosis in all systems. However, there is increasing evidence that a caspase-independent pathway does exist. Even in the classical model of neuronal death, induction of motoneuron death by limb-bud removal is founded to be caspase independent, whereas naturally occurring motoneuron death has been shown to depend on caspase-3 (Milligan et al., 1999). However, the novel caspase independent pathway(s) is/are not fully understood and remain(s) to be characterised.

Cellular Regulation of the PCD and the Neurotrophic Hypothesis

The biological roles of PCD in neuronal populations are still not clear. Many possibilities have been proposed. Some examples of these are:

1) Neuronal death may eliminate defective cells.

2) Cells die to shape the gross morphology of regions of the nervous system.

3) Cells die to create space and permissive pathways for axon outgrowth.
4) The early neurons might serve or mediate transient functions in development (e.g. as temporary targets for early afferents) but die later when functions are no longer needed.
5) Death of neurons might also correct erroneous projections.
6) It is also possible that cells may die to limit or control cell number (McConnell et al., 1989; Oppenheim, 1991; Burek and Oppenheim, 1998; Allendoerfer and Shatz, 1994).

Whether or not these hypotheses are true remains to be investigated.

The mechanisms regulating PCD in all these possible roles seem to involve trophic factor supply and cell-cell interaction (either synaptic or non-synaptic). This implies that PCD in neuronal populations is likely to be under epigenetic control (although genetic programming can not be excluded). At the tissue level, targets, afferents, and interaction with neighboring cells (neurons or glia) can control cell death (Cowan et al., 1984). In the following discussion, I shall focus principally on the regulation of cell death by interaction with the target.

Targets are strongly implicated as a major influence in regulating survival. These effects are proportional, e.g. the size of the target can determine the amount of PCD. This strongly supports the hypothesis of a role of PCD in the control of cell number and target matching (Oppenheim, 1985). One example comes from the studies in motoneuron death in chick embryos (Oppenheim, 1981; Oppenheim et al., 1978). 50% of chick motoneurons undergo PCD between embryonic day (E)10-12, the stages that neuronal survival and death are dependent upon target-derived signals (Oppenheim et al., 1982). Removal of their natural target, limb-bud, also increases neuronal death up to 100% (Hamburger, 1934).

In fact, similar findings had been found earlier in sensory and sympathetic ganglia (Levi-Montalcini and Hamburger 1949; 1951). Later, in 1957, these workers discovered the first characterised neurotrophic factor – nerve growth factor (NGF) (see review in Levi-Montalcini, 1987). The correlation between the survival role of

target influences and the discovery of trophic molecules led to the emergence of **the Neurotrophic Hypothesis**. The hypothesis has been proposed to explain PCD around the period of target innervation. The essence of the hypothesis is that 1) neurons are initially overproduced and become dependent on targets later in development; 2) in order to get rid of overproduced and inappropriately connected neurons, the targets produce a **limited supply** of neurotrophic molecules; 3) the innervating cells have to **compete** for the neurotrophic molecules in order to survive; 4) neurons which fail to obtain sufficient trophic support will undergo PCD.

Since the role of NGF was originally identified in sensory and sympathetic neurons, this hypothesis has become firmly established in the peripheral nervous system (PNS) (Levi-Montalcini and Hamburger, 1957). It is still not known whether this notion applies to the whole nervous system (although many have assumed it to be generally true in all cases). The central nervous system (CNS) is generally more complicated than the PNS. Thus, it is possible that central neurons in the brain might use other trophic factors, different mechanisms, or even use PCD for different purposes. The later discovery of an NGF related protein, brain-derived neurotrophic factor (BDNF), in the brain (e.g. Barde et al., 1982) further supports the idea that the neurotrophic hypothesis may apply in the CNS. However, in certain core CNS structures such as the thalamus, convincing evidence of PCD and the neurotrophic hypothesis have not been clearly demonstrated.

Increased cell death following deafferentiation has been reported in several areas in the nervous system such as in chick cochlear nucleus (Levi-Montalcini, 1949) and in the ishmo-optic nucleus (Clarke, 1985). Glia-derived support, extracellular matrix molecules, and local cell-cell interactions have been shown to promote the survival of developing neurons elsewhere (see Hamburger et al., 1981, Barde, 1989). However, when compared to the role of target, these influences are considered to be less prominent (Oppenheim, 1985).

Neurotrophin Signaling and its Regulation in Programmed Cell Death

Trophic factors do more than just control cell survival, and their importance is not restricted only to embryogenesis. Their functions are diverse. Some other prominent roles are in proliferation, synaptic plasticity, cellular metabolism, axonal growth, differentiation, and regeneration after injury (see review by Lewin and Barde, 1996). The factors are also important in general adult physiology, in pathological conditions, as well as in development but this does not mean that they are simply ubiquitous metabolites necessary for cellular maintenance and growth. They have tissue specificity, time specificity (i.e. they may function only at a particular stage of the development), and act via specific signaling pathways (Davies, 1988a; b).

In the nervous system, the best-known trophic factors regulating cell survival are members of the neurotrophin family. Neurotrophins are required for the survival and differentiation of specific neuronal populations during ontogeny. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin 4/5 (NT-4/5) and neurotrophin 6 (NT-6) are members of the family. The receptors for these molecules consist of three high affinity receptors called tyrosine kinase receptors (TrkA, TrkB and TrkC) and the p75 low affinity receptor. NGF binds to TrkA, BDNF and NT-4/5 bind to TrkB, NT-3 binds to all three Trks (Figure 1.5). All neurotrophins also bind to the p75 receptor (Meakin and Shooter, 1992; Barbacid, 1994). The receptor for novel NT-6 is still unknown and its function in mammals has never been found. TrkB and TrkC exist as isoforms. TrkB has at least three isoforms, one catalytic with a tyrosine kinase domain, and two non-catalytic (Klein et al., 1990) isoforms. TrkC comprises at least three catalytic and two noncatalytic isoforms. Despite the similarities between the neurotrophins, cross-talk between some of these factors and their non-preferred receptors is rare, with the only exceptions occurring on application of excessive and non-physiological amounts of the trophic factors (Thoenen, 1991). All these neurotrophin receptors (both Trk receptors and p75) control PCD of developing neurons (Carter and Lewin, 1997), but Trks and p75 may have antagonistic effects (Carter and Lewin, 1997).

Neurotrophins signal via both tyrosine kinase receptors and the p75 receptor and regulate several cellular activities including apoptosis (Segal and Greenberg 1996). The review of the known intracellular pathways is as follows.

A. Activation through tyrosine kinase pathway

This is a common pathway used also by several other traditional growth factors such as fibroblast growth factors (FGFs) and platelet-derived growth factor. Once a neurotrophin binds a tyrosine kinase receptor, the receptor dimerises with another receptor and is phosphorylated. This activated dimer then can transduce the signals through many intracellular pathways. A major pathway for cell metabolism is via a proto-oncogene, Ras. The other, which is less understood, may be termed the Ras-independent pathway.

1) Ras-dependent pathway:

1.1) Ras-MAP Kinase pathway: This pathway induces neurite outgrowth and cell proliferation by activation of several immediate early genes (IEGs e.g. c-fos, c-jun, cyclin D1).

1.2) Ras dependent Phospholipase C gamma (PLC-γ) pathway: The activated PLC-γ cleaves phosphatidyl inositol 4,5 bis phosphate (PIP4,5) to diacylglycerol (DAG) and inositol triphosphate (IP3), and activates MAPK.

2) Ras-independent pathway:

2.1) PI3 kinase: it is responsible for cell survival, preventing apoptosis by acting through protein kinase B (the protein also known as AktK), which keeps *Bad* phosphorylated and hence dissociated from *Bcl-xL*.

2.2) SNT*[1] Pathway: by an unknown mechanism, this induces the cell to exit from the cell cycle. This pathway is characteristic only to neurotrophins (NGF, BDNF, and

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[1] ***suc1***-associated neurotorphic factor-induced tyrosine-phophorylated target (Rabin et al., 1993)
suc1: yeast's (*Schizoachharomyces Pombe*) sucrose fermenting protein 1, a protein associated with cell cycle (Carlson et al., 1981; Halyes et al., 1986).

NT-3), and is not present in any other tyrosine kinase activation by other related growth factors (such as EGF, PDGF, and FGFs).

B. Activation through the p75 receptor, the relative of the TNFα receptor (Carter and Lewin, 1997):

Because of its low affinity for the neurotrophins and its lack of specificity in binding with all neurotrophins, this receptor was thought to be less important for neurotrophin function. However, later studies have shown its significant roles in neurotrophin signaling. Firstly, by unknown mechanisms, this receptor enhances Trk signaling by facilitating Trk dimerisation (Barker and Shooter, 1994) and stimulating tyrosine phosphorylation (Verdi et al., 1994). Additionally, in a more prominent function, p75 acts as a death receptor (Casaccia-Bonnefil et al., 1996). This receptor activates the sphingomyelinase pathway resulting in the increased production of ceramide. Another pathway involves the activation of a transcription factor NFkB. Downstream mechanisms by which both ceramide and NFkB induce apoptosis are still not clear, but are thought to involve the activation of a Bcl-2 family member (Greenlund et al., 1995), kinases, and proteases including caspases (Carter and Lewin, 1997).

However, the death receptor function of p75 is found only in NGF signaling. Neither BDNF nor NT-3 (the two main neurotrophins in the central neurons) activates apoptosis by this pathway. Furthermore, p75 is unlikely to control PCD (Oppenheim, 1999). p75 was not my focus in the studies of PCD in the CNS.

The signaling of the neurotrophins is summarised in Figure 1.3.

Nonetheless, some other neurotrophic factors such as CNTF (Ciliary neurotrophic factor) use a different system for their signaling. As CNTF is related to interleukin-6 (IL-6) and the leukemia inhibitory factor (LIF), it utilises a cytokine receptor system named the Jak-Tyk kinases system. The detail will not be discussed here since there is no direct evidence of a role of this factor in PCD or development in the thalamus (Ip and Yancopoulous, 1996).

Critical Period & Neurotrophic Switch

A striking feature coinciding with PCD in sympathetic neuroblasts is the switching in responsiveness to neurotrophins. Initially, around E15, these cells are strongly NT-3 dependent, and do not respond to NGF *in vitro*. By birth, on the other hand, these neurons become dependent only on NGF (Birren et al., 1993; Dechant et al., 1993). High doses of NT-3 promote only poor survival (Birren et al., 1993; Dechant et al., 1993). This change in neurotrophin-responsiveness corresponds with a change from expression of *trkC* to that of *trkA* mRNA in the cells *in vivo* (Birren et al., 1993). The time of this switch occurs around the time of target-innervation.

Similar findings of a switch in trophic requirements have been made in other neuronal tissues. Extensive research led by Davies shows that neurons of the trigeminal ganglion, vestibular ganglion, and jugular ganglion undergo a trophic switch (see Davies, 1994a; b; Pinon et al., 1996). Later, he correlated the neurotrophic hypothesis with the trophic switch findings. He proposed that cell death during target innervation is due to the switch in trophic factor dependence (Davies, 1988; 1996; 1997). The suggestions of this new proposal, modifying the neurotrophic hypothesis, are as follows. 1) Initially neurons are dependent on certain trophic factor(s) before they innervate their targets. The factor(s) are either relatively redundant or are present in most tissues and are generally required for cell survival. 2) Later, some intrinsic program switches the trophic responsiveness of the neurons to a new trophic factor, which is not present in their own tissue. 3) The cells need to find the correct target to obtain the new trophic support. 4) The control of neuronal PCD may be by competition for target-derived trophic factors in limited supply, or some neurons may lose the ability to respond to the new factors.

Nonetheless, not all developing neurons switch their neurotrophic dependence. For example, Buj-Bello et al. (1992) found that nodose neurons respond to both NT-3 and BDNF throughout their life. Vestibular neurons depend only on

BDNF. Unlike PCD or the neurotrophic hypothesis, the trophic switch seems not to be a universal phenomenon.

The trophic switch may not apply to all nervous tissues. However, this switching hypothesis may provide us with a better understanding of the mechanism underlying PCD. The upregulation of neurotrophic factor receptors that is found in all cases where switching occurs correlates with the target innervation period. This supports the neurotrophic hypothesis (reviewed in Davies, 1996), i.e. it suggests that the target innervation period is a critical period and cell survival during this time is regulated by neurotrophins. Since most of the studies of this switching event were done in ganglionic neurons, we do not know whether trophic switching occurs in central neurons.

Programmed Cell Death and the Neurotrophic Hypothesis in the Brain

PCD has been reported to occur in virtually every developing nervous tissue (Oppenheim, 1991). In the brain, PCD has been found in many regions of many vertebrate species such as the cerebral cortex (reviewed by Ferrer et al., 1992), the ishmo-optic system (Clarke, 1985), and the retino-tectal system (reviewed by Holm and Isacson, 1999).

Yet, only few investigations on PCD have been carried out in the thalamus. This tissue is an important structure in the CNS and has the cerebral cortex as its major target. It is a core component of the forebrain lying within the diencephalic wall, which collects and feeds not only sensory but also motor information from several regions of the nervous system to the cortex.

Most studies of PCD in thalamus are limited to the dorsal lateral geniculate nucleus (Sengelaub et al., 1985; Finlay and Pallas, 1989) and the ventrobasal complex (Waite et al., 1992) and are based on conventional histological techniques. Although they provided good evidence of PCD in these regions of the thalamus, the results do not necessarily represent PCD in the rest of the regions of the thalamus.

Spreafico et al. (1995) attempted to examine PCD in both cortex and thalamus using a more sensitive technique – TUNEL (*T*dT-mediated *dU*TP-biotin *n*ick *e*nd *l*abeling) - but failed to analyse the actual amount of cell death in the thalamus. To date, we still do not know the precise time, specific location and quantity of PCD in this area. As PCD in the thalamus is poorly characterised, the likely applicability of the neurotrophic hypothesis in this region is still ambiguous. The influences of the target, cerebral cortex, on thalamus are documented for thalamic axonal guidance and outgrowth (Lotto et al., 1994; Hubener et al., 1995), but the roles of this target in regulating PCD in the thalamus are not fully understood.

Factors that Control Thalamic Programmed Cell Death

Both intracellular and extracellular signals can regulate PCD, but we do not know what regulates thalamic survival during development. As reviewed in the beginning of this chapter, the survival of many sensory, motor and sympathetic neurons depends on the presence of neurotrophic factors (Altman, 1992). According to the neurotrophic hypothesis, it is plausible that thalamic cells also depend on neurotrophic factors, and it is also possible that the required neurotrophic factors are target (cortical) derived.

However, only a few studies support the idea of a survival role for trophic factors in thalamus and the relevance of the neurotrophic hypothesis, that target support is pivotal for thalamic survival, is unclear. Hisanaga and Sharp in 1990 showed that diffusible substances from cerebellar cortex and neocortex have neurotrophic effects on survival of thalamic cells (Hisanaga and Sharp, 1990). In 1996, Lotto and colleagues showed that neurotrophins (NTs) and members of the fibroblast growth factor (FGF) family enhance thalamic survival *in vitro* (Lotto et al., 1996). Unfortunately, we still do not have enough information to conclude that any particular trophic factors do regulate thalamic survival *in vivo*.

Evidence that Neurotrophins Might Play a Survival Role in Developing Thalamus

The most prominent candidates for regulating thalamic survival are neurotrophins. Supporting evidence comes from studies of the expression of neurotrophins and their receptors. *trkA* is expressed at low level in the brain including the thalamus throughout the animal's life. High levels of *trkB* and *trkC* expression in some thalamic nuclei suggest the physiological significance of their ligands in the thalamus (Ringstedt et al., 1993). Since NGF and NT-4 levels are usually low while BDNF and NT-3 levels are high in the brain, the latter two are more likely to play a role in thalamic development. Neurotrophins might play many roles in thalamic development, but in my studies, I have focussed on their role in the regulation of PCD.

The Trk molecules are normally expressed very early in development. For example, *trkB* and *trkC* mRNA can be detected in brain from E8-E10 (Klein et al., 1990). The expression of the neurotrophins and their receptors during development are spatio-temporally regulated. This implies that if neurotrophins regulate cell survival in the brain, they must act only in a particular period. In rat thalamus, *trkB* and *trkC* are expressed strongly in certain nuclei in the first postnatal week but expression declines gradually by 2 and 4 weeks old (Ringstedt et al., 1993). Nevertheless, their expression patterns in the prenatal thalamus have not been defined in detail.

To understand the physiology of these molecules, mutant mice lacking neurotrophins and their receptors were generated; *trk* (the gene encoding for TrkA receptor) knockout by Smeyne et al. (1994), *trkB* knockout by Klein et al. (1993), *trkC* knockout by Klein et al. (1994). It is interesting that in many cases, neurotrophin knockouts show only limited defects when compared to the knockouts of their neurotrophin receptor counterparts (Alcantara et al., 1997). For instance, double mutant mice lacking *BDNF* and *NT-4* fail to reveal motor neuron deficits (Conover et al., 1995; Liu et al., 1995) while *trkB* knockouts show a significant

decrease in motor neuron numbers (Klein et al., 1993). This might be a consequence of compensatory effects or trophic redundancy (Johnson and Oppenheim, 1994). This idea is also supported by the work of Silo-Santiago and colleagues in 1997 showing that double *trkB* and *trkC* knockouts have more severe sensory deficits in development than *trkB* or *trkC* knockouts alone. Thus, the knockout studies might provide useful information on the regulation of PCD during development in the thalamus.

Review: Anatomy and Development of the Thalamus

Thalamus functions as the nervous system relay station

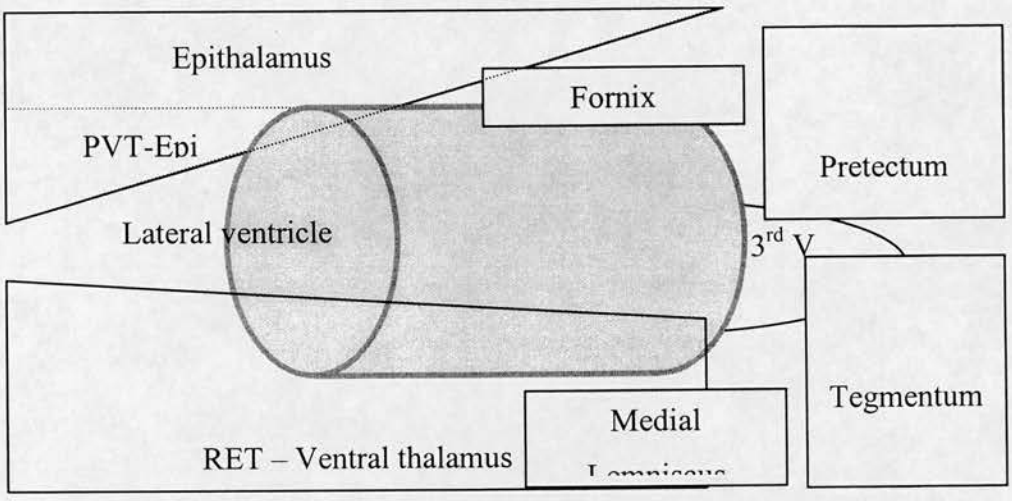
In order to gain a better knowledge of PCD in the thalamus, some basic knowledge on this structure should be reviewed. The thalamus is divided into three parts; epithalamus, dorsal thalamus, and ventral thalamus. Only dorsal thalamus directly projects axons to cerebral cortex. Almost all sensory and motor information going to the cerebral cortex is processed and distributed by the dorsal thalamus (Kandel et al., 1991). The functions of both structures are, therefore, interdependent. The thalamus is also involved in autonomic reactions and the maintenance of consciousness in conjunction with the limbic system. Since my interests are in the relationship between the thalamus and the cerebral cortex, the term 'thalamus' as used in later chapters of my thesis refers to the dorsal thalamus.

The major outflow of the dorsal thalamus is the thalamocortical projection. Jones (1985) showed that every dorsal thalamic nucleus projects to the cerebral cortex. Some fibres project only to the cerebral cortex while others project to the cortex and branch to other basal regions of the cerebral hemisphere. These branches give rise to thalamostriatal and other small thalamic projections.

Thalamus: anatomical description and subdivision (in rodents)

Anatomically, the thalamus refers to bulging diencephalic masses lying between the lateral ventricles and the third ventricle. The boundaries of the dorsal thalamus are as follows. Most of the anterior surface is covered by the reticular nucleus (a part of the ventral thalamus) which, more posteriorly, becomes the ventral and ventro-lateral bounderies. The medial lemniscus fiber also lies as the posterior part of the ventral boundary. The more medial part of the anterior surface is covered by paraventricular nucleus of the epithalamus. The upper borders of the dorsal thalamus are the third ventricle, epithalamus and pretectum. The pretectum and the tegmentum then form the posterior boundary. The upper lateral surface is the lateral

ventrical and fornix (or may be exposed to the intracranial cavity during development). The medial surface of the thalamus either contacts with the other thalamus or with the third ventricle (Jones, 1985; Schambra et al., 1992; Paxinos et al., 1994; Warren, 1996). The ventral thalamus and the epithalamus are divided from the dorsal thalamus by two thin cell sparse zones (Edgar, 1998).



(Diagram shown the position of the thalamus (gray object) relative to the neighboring structures)

The dorsal thalamus is subdivided into several nuclei. These subdivisions are particularly difficult to visualise in three dimensions. Depending upon their fiber connections, most of the major thalamic nuclei can be classified into two groups. The first group relays inputs from individual sensory and motor systems and comprises so called specific relay nuclei, whereas the association nuclei, on the other hand, have widespread connections and diffuse-projections (e.g. intralaminar nuclei). The anatomy of the thalamic nuclei is shown diagrammatically in Figure 1.6 and their functions are summarised in Figure 1.7 (modified from Kandel et al., 1991).

All anatomical divisions are derived from Jones (1985), Carpenter (1991), Schambra et al. (1992), and Paxinos et al. (1994). The detailed description of each nuclear group is as follows.

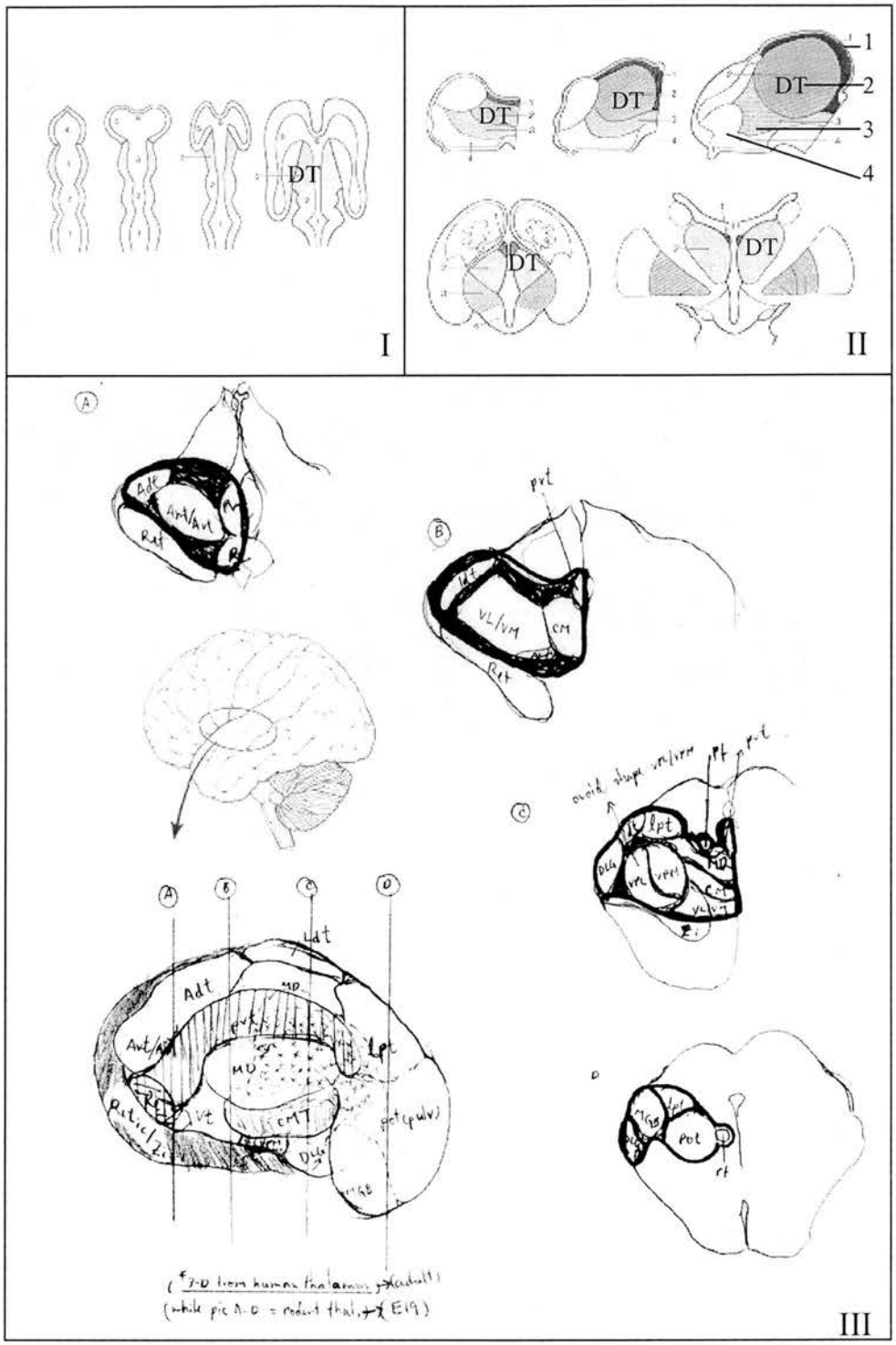
Figure 1.5

Developing and mature thalamus: Sequential developing diencephalon (shaded), the region giving rise to the thalamus, is shown in (I). Formation of the thalamus (in sagittal and transverse planes, II) follows the extensive changes in each structure and positions due to the differences in regional growth (1 = Epithalamus, 2 = dorsal thalamus, 3 = ventral thalamus, and 4 = hypothalamus). The left transverse plane shows thalamus of an embryonic brain. The right shows the adult thalamus.

Picture III represents of a human thalamus in 3-dimensions (as viewed from the medial wall of the 3rd ventricle). Analogous transverse planes (A-D) of E19 mouse thalamus are depicted from anterior to posterior.

Abbreviations: Adt = Anterodorsal thalamic nucleus. Amt/Avt = Anteromedial and anteroventral nuclei. VL/VM = ventral lateral and ventral medial nuclei, CM = Centromedial nucleus, ldt = lateral dorsal nucleus, MD = mediodorsal (or dorsomedial) nucleus. Lpt = lateral posterior nucleus, VPL/VPM = Ventral posterior (ventrobasal) lateral and ventral posterior medial nuclei, DLG = dorsal lateral geniculate nucleus, MGB = Medial geniculate body (nulceus), PoT = posterior nuclei, Pvt = Paraventricular nuclei (epithalamus, Ret = Reticular nucleus (ventral thalamus), Zi = Zona incerta (ventral thalamus), pf = fasciculus retroflexus and the parafascicular nucleus.

(Kandel et al 1991, Kahle 1993, personal camera lucida drawings)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1) Anterior nuclear group i.e. anteroventral (AV), anteromedial (AM), and the anterodorsal (AD, which extends to form lateral dorsal nuclei - LD). These nuclei (except LD) lie in the most rostral part of the dorsal thalamus. AD is easy to visualise by its wedge-shape just beside (medial to) the stria medularis. They relay limbic information, particularly emotion.

2) Ventral nuclear group consist of the ventral (ventral lateral and ventral medial, VL/VM), and the ventral posterior nuclei (VP, or ventrobasal complex which is subdivided to ventroposterior medial and ventroposterior lateral). Ventral nuclei are recognised by their loosely packed cells lying medially and more anterior to the ventral posterior nuclei but behind the anterior nuclear group. Ventral posterior nuclei have a distinctive appearance, being lobulated in shape.

3) Posterior nuclei (PoT) are anterodorsal to the medial geniculate body (MGB) and anteroventral to VPL. Together with MGB, they form the most dorsal part of the dorsal thalamus.

4) Lateral posterior nuclei (LP) (including the pulvinar, which is not seen in rodents) is usually dorsal to the VP, under LD and VL, between DLG and PoT.

5) Medial Geniculate body (MGB) is the caudo-ventral part of the dorsal thalamus. It lies lateral to the brachium of the inferior colliculus. These nuclei mediate hearing information.

6) Lateral Geniculate body (LGB) is perhaps the most prominent in primates. However, in rodents it is small and much flatter. It lies lateral to the ventral nuclear group and ventral to the LP/LD. Only the dorsal part (DLG) of the lateral geniculate body derives from the dorsal thalamus. These nuclei relay information for the visual system.

7) Medial dorsal group and the intralaminar nuclei (e.g. midline nuclei, centromedian): most of these nuclei lie along the medial wall of the dorsal thalamus. They have both diffuse projections and limbic function. Moreover the mediodorsal nuclei also have extra specific projections to prefrontal cortex. These nuclei modulate the activity of both the cerebral cortex and the thalamus itself.

Figure 1.6

Summary of major connections and functions of thalamic nuclei (Kandel et al., 1991)

Connections and Functions of Thalamic Nuclei

Nuclei	Principal afferent inputs	Major projection sites	Function
Relay nuclei			
Anterior nuclear group	Mammillary body of hypothalamus	Cingulate gyrus	Limbic
Ventral anterior	Globus pallidus	Premotor cortex (area 6)	Motor
Ventral lateral	Dentate nucleus of cerebellum through brachium conjunctivum (superior cerebellar peduncle)	Motor and premotor	Motor
Ventral posterior			
Lateral portion	Dorsal column–medial lemniscal pathways and spinothalamic pathways	Somatic sensory cortex of parietal lobe	Somatic sensation (body)
Medial portion	Sensory nuclei of trigeminal nerve (V)	Somatic sensory cortex of parietal lobe	Somatic sensation (face)
Medial geniculate	Inferior colliculus through brachium of inferior colliculus	Auditory cortex of temporal lobe (areas 41 and 42)	Hearing
Lateral geniculate	Retinal ganglion cells through optic nerve and optic tract	Visual cortex (area 17)	Vision
Lateral dorsal	Cingulate gyrus	Cingulate gyrus	Emotional expression
Lateral posterior	Parietal lobe	Parietal lobe	Integration of sensory information
Pulvinar	Superior colliculus, temporal, parietal, and occipital lobes	Temporal, parietal, and occipital lobes	Integration of sensory information
Medial dorsal	Amygdaloid nuclear complex, olfactory, and hypothalamus	Prefrontal cortex	Limbic
Diffuse-projection nuclei			
Midline nuclei	Reticular formation and hypothalamus	Basal forebrain	Limbic
Intralaminar, centromedian, and centrolateral nuclei	Reticular formation, spinothalamic tract, globus pallidus, and cortical areas	Basal ganglia and cortex	
Reticular nucleus	Cerebral cortex and thalamic nuclei, brain stem	Thalamic nuclei	Modulation of thalamic activity

Development of the thalamus and thalamocortical connections

Like the development of all other neuronal tissues, the early formation of the thalamic mass is achieved through the processes of proliferation and differentiation. Emerging from the diencephalon, the thalamus is formed around E11 and E15 in mouse (Angevine, 1970; Rennie, 1993). The gradient of its proliferation occurs ventrodorsally (from bottom to top), caudorostrally (from back to front), and lateromedially (from outside-in) (Angevine, 1970). Each thalamic nucleus has its individual period for the peak of cell proliferation, which on the average occurs around E14. By E17, most of the major thalamic nuclei are formed and discernible. By the time just before birth, production of neurons and formation of the nuclei are almost completed (Altman and Bayer, 1988).

With regard to the establishment of the fiber connections, the innervation of the thalamus is an early event. Many inputs including spinal tracts and trigeminal tracts reach the thalamus between E15-E17 (Leamey and Ho, 1998). Corticothalamic fibers arrive at the thalamus around the stage of thalamic proliferation but before thalamocortical fibers innervate cortex (Jones, 1985). Thalamocortical fibers, the major output of the thalamus, extend their axons to the internal capsule between E14 and E15 in mice. After E17, the fibers begin their radial innervation into the cortex. During this time, a transient structure called the subplate zone in the cortex provides a temporary target for these axons (Rakic, 1977; Chun and Shatz, 1989). Shortly after birth, most of the thalamic fibers have terminated within layer 4. Coinciding with this period, some cells in the lateral geniculate nucleus of the thalamus undergo PCD (Finlay and Pallas, 1989).

Possible Role of Developmental Genes that Regulate Thalamic Neuronal Survival

Some genes upstream of the cell death machinery can directly control apoptosis. As we have already seen, genes encoding for transcription factors, *ces-1* and *ces-2* regulate developmental cell death in nematodes. The sonic hedgehog (Shh) gene encoding for a glycoprotein that is expressed in the notochord and floorplate, also has been shown to promote the survival of fetal ventral spinal and mesencephalic neurons in culture (Miao et al., 1997). Recent evidence has also shown that this gene regulates the survival of chick spinal cord neurons *in vivo* (Oppenheim, 1999). Although the precise mechanisms by which these genes control cell death are still not known, the PCD pattern correlates well with their spatio-temporal expression pattern in the spinal cord.

In the later part of my thesis, I have looked at the possible role of a developmental gene, *pax-6*, and whether it has a direct or indirect role in cell death regulation. *Pax-6* is a transcription factor gene expressed in the CNS, eye and nose (Quinn et al., 1996). In the forebrain, *pax-6* expression is found throughout the entire prosencephalon at around E9.5 but becomes more restricted only to the telencephalic ventricular zone and the ventral thalamus by E14.5 (Stoykova, 1996). This spatio-temporal pattern of *pax-6* expression suggests that it has an important role in developmental processes. In mice carrying a point mutation of the *pax-6* gene – *Small-eye (Sey)* mice – many brain structures are abnormal. Cell proliferation, cell number, and some axons such as those of thalamocortical tracts (Kawano et al., 1999; Edgar, 1998) are severely disrupted in homozygous *Sey/Sey* mice. These homozygous mice do not survive after birth.

Interestingly, even though *pax-6* is not expressed in the dorsal thalamus, its morphology is severely affected by the mutation of this gene. The dorsal thalamus in the mutant is very small and its border is less clear as the embryo develops (Edgar, 1998). The crucial role of this gene in the dorsal thalamus has yet to be determined. One of the explanations for the abnormal morphology in the dorsal thalamus when

investigate at the early stages (E10.5-E14.5) is that it is caused by proliferation defects rather than by an increase in cell death (Warren and Price, 1997). However, cell death in this mutant after the period of innervation has not been investigated.

Grindley et al. (1995) showed that this mutant has an increase in cell death in the nasal placode region at around E10. However it is still unclear whether this death correlates with a direct survival role of the gene. Since there is thalamocortical fiber disruption in *Sey/Sey* mice, according to the Neurotrophic Hypothesis I predicted that there should be an increase in cell death in the dorsal thalamus at late gestation (i.e. during the target innervation period) in this mutant. This would imply an indirect role of the *pax-6* gene in regulation of cell death, secondary to its effects on thalamocortical axons.

Studying Apoptosis in the Developing Thalamus:* in vitro *and* in vivo *Approaches

Apoptosis was first characterised morphologically by using conventional histological methods and electron microscopy. The simplest *in vivo* method involves haematoxylin staining (Ramachandra and Studzinski, 1995) or Nissl staining (Oppenheim, 1986). The dyes stain the nucleus of every cell but will pick up hyperchromatically the condensed nuclei of cells in some states such as mitosis, apoptosis, and the pyknotic phase of enucleating red blood cells (during development). Differentiating apoptotic cells from other densely stained cells (mitotic and enucleating red blood cells) by these methods sometimes can be difficult, especially during development. To overcome this problem, Gavrielli et al. (1992) developed TUNEL for *in situ* detection based on the DNA fragmentation characteristic of apoptosis. This TUNEL method has now become a gold standard for detection of apoptosis not only *in vivo* but also *in vitro*.

There are other means apart from morphological/histological ones for the detection of apoptosis. These include cytological and biochemical methods, and immunoblotting in caspases assays. Flow cytometry (cytological) and DNA

laddering detection in gel electrophoresis (biochemical) are useful for quantitative analysis (Eisel et al., 1998). But these techniques cannot identify the precise location where the apoptosis actually take places. Antibodies to caspases have a similar problem and they have not been successful in histochemical applications. Moreover, apoptosis may also occur via a caspase independent pathway and the use of caspase detection is limited.

Although the advantage of *in vivo* studies is that they allow the actual event occurring in development to be studied, there are some limitations that prevent us from understanding the underlying processes. One of the main constraints on studying apoptosis *in vivo* is the rapid rate of elimination of apoptotic cells by phagocytes such as macrophages (or microglia in the CNS) (McGahon, 1995). In addition, methods for experimental manipulation are limited in living animals.

Primary cell cultures allow us to define and vary the biological components of an experiment. The cells stay in a more controllable environment and it is easier to measure or observe the effects of experimental stimuli (Johnson, 1995). Serum-free tissue culture has long been used for studies of the trophic effects of added molecules (Lotto, 1995), because it excludes the huge amount of other non-specific trophic molecules or metabolites existing in the blood which are likely to mask the effects of the molecules under study. This technique is useful for observing the direct effect of neurotrophic factors on cell survival. One disadvantage of primary cell cultures, like *in vivo* studies, is that they contain a heterogenous population. This is not a major issue in this thesis since thalamic cells used in here were at the ages (E15-P2), when the thalamus has been shown to contain mainly neurons (Lotto, 1995; Lotto et al., 1997).

Measurement of Cell Viability **in vitro**

There are several methods for assaying cell death and viability *in vitro* (see summarised illustration in Figure 1.7. (Figure obtained from Johnson, 1995). Some

Figure 1.7

Examples of available methods for measurement of cell death: Detail is described within the figure (Johnson, 1995)

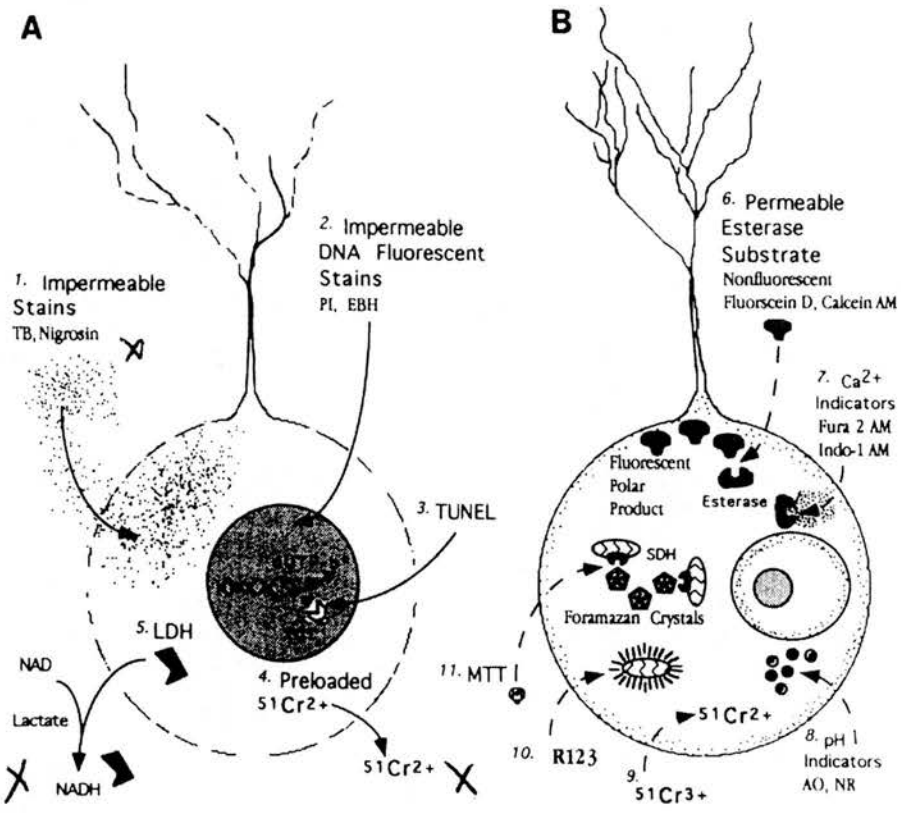


Fig. 1 An overview of some of the labeling and quantitative methods that are described in this chapter for assays of cell death and viability *in vitro*. (A) Cytotoxicity Assays. 1. Membrane impermeable colloidal stains that are normally excluded by living cells can readily pass through damaged membranes of dead cells to stain cytosolic proteins. TB (Trypan blue) and Nigrosin are examples. 2. Fluorescent DNA intercalating dyes that are normally membrane impermeable can rapidly stain the nuclei of dead and dying cells. PI (propidium iodine) and EBH (ethidium homodimer 1) are examples. 3. DNA fragmentation can sometimes be detected during apoptosis by *in situ* end-labeling with biotinylated nucleotides. TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUTPO–biotin end-labeling] can detect these events. 4. Cultured cells can be preloaded with salts containing reduced radioactive chromium. The oxidation of chromium yields a normally membrane impermeable form retained by living cells. However, during cell death there is an increased leakage of radioactivity into the media. 5. Cell death can be monitored with automated assays that detect the presence of normal cytosolic enzymes released into the culture media. LDH (lactate dehydrogenase) is one such marker. (B) Viability assays. 6. Nonfluorescent analogs of fluorescein are membrane permeable. They can be converted by cytosolic esterase enzymes to polar fluorescent compounds that are retained by living cells. Fluorescein diacetate and calcein AM (acetoxymethyl esters) are examples. 7. Changes in intracellular free calcium can be measured in living cells with permeable. AM (acetoxymethyl ester) analogs of the calcium ion indicators fura-2 and indo-1. 8. Proton pumps maintain the acidic environment of some organelles (i.e., lysosomes) in living cells. Their activity can be labeled with pH-sensitive fluorescent dyes and stains such as AO (acridine orange) and NR (neutral red). 9. Membrane permeable salts of reduced chromium can be used to assay the retention of radioactivity following chromium oxidation by living cells. Leakage of oxidized chromium can also be used as an assay of cell death (see 4). 10. R123 (Rhodamine 123) is a potential sensitive fluorescent dye specific for mitochondria in living cells. 11. The mitochondrial enzyme SDH (succinate dehydrogenase) reduces MTT (a soluble tetrazolium salt) into water insoluble blue formazan crystals in living cells.

of the widely used methods are reviewed below (Schwartz, 1995; Schwartz and Osborne, 1995).

1. Method based on mitochodrial activity: Mitochondrial enzymatic assay (MTT assay)

This colorimetric assay is used to determine viability of cells in microplate cultures (Mosmann, 1983). A tetrazolium substrate – 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) – is reduced by the enzyme succinate dehydrogenase within the active mitochondria of living cells. The substrate is converted to an insoluble dark blue formazan product in living cells which can be quantified by using an automated ELISA microplate reading machine. Less viable cells convert less substrate. However the quantitation is sensitive to plating density changes. And it cannot tell us the actual number of cells undergoing cell death. Moreover the technique determines only an overall viability of cultures but cannot differentiate apoptosis from necrosis.

2. Detections based on the membrane properties:

2.1) Membrane lipid alteration detection

This is another method commonly used in flow cytometry that can only be used to stain prefixed cells. It is based on the flip-over of phosphatidyl serine (PS), which is normally on the inner side of the membrane, to the outer side of the cell membrane in apoptotic cells (Fadok, 1992). Annexin V can then bind and detect the exposed PS very specifically and rapidly (Eisel et al., 1998).

2.2) Membrane integrity staining

Propidium iodide (PI) and ethidium bromide (EtBr) are two exclusion dyes commonly used to test membrane integrity. Healthy cells and early apoptotic cells will normally have their membrane intact and thus do not take up the dyes. Both dyes fluoresce at the rhodamine wavelength and can be used in combination with other

UV or fluorescein excitation dyes such as Hoechst, Acridine Orange and SYTO-13 (Sun, 1992; Ben-Sassen, 1995; Coligan et al., 1995; Crissman, 1995).

However none of the membrane testing methods alone can differentiate late stage of apoptosis and necrosis (which cells are also highly permeabilised).

3. Detection based on nucleus properties.

3.1) TUNEL detection

This method can be used with a colorimetric peroxidase reaction or with fluorescence. It is based on specific DNA fragmentation presented in apoptotic cells. The detail will be discussed in the next chapter. The application of the technique is both *in vivo* and *in vitro*. But it is only useful to detect apoptosis in particular, it is labor-intensive, time-consuming, and costly.

3.2. Fluorochromes staining for study of chromatin

Dyes used for staining chromatin structure include acridine orange (AO - metachromatic fluorescent), SYTO-13 (green fluorescent), Hoechst 33342 (blue in UV filter) and DAPI (blue in UV filter). They enter all cells and bind DNA. Apoptotic cells are stained characteristically with these dyes (Abrams et al., 1993). In thalamic cultures, all neurons were labelled and the prominent pyknotic cells were brighter stained (Lotto, 1995). These dyes are useful for observing nuclear changes of cells undergoing apoptosis.

4. Combination staining

To overcome the limitations of each technique for studying cell viability, many have proposed a way to combine dyes that stain nucleic acids with membrane exclusion dyes to provide a simple and rapid method to visualise cell death (Darzynkiewicz et al., 1992; Coligan et al., 1995; Schwartz, 1995; Schwartz and Osborne, 1995). The use of chromosomal dyes (for example, blue UV emitting

Hoechst dye) with one of the membrane impermeant dyes such as ethidium bromide or propidium iodide is an excellent combination for cell viability examination (Darzynkiewicz et al., 1992; Sun, 1992). One advantage of this combination is that rhodamine dyes can also fluoresce as red in other filters, making both dyes visible with one filter (i.e. EtBr is seen in UV filter if combined with Hoechst).

According to the previous study with this combination technique, Coligan and colleagues have classified cells into 4 groups (Coligan et al., 1980). 1) Living cells have normal looking Hoechst positive nuclei (blue). 2) Necrotic (pathologically dead) cells are stained red (due to the entry of EtBr or PI) and have relatively normal size. 3) Early- apoptotic cells are cells displaying blue pyknotic nuclei 4) Late-apoptotic cells have red pyknotic nuclei.

In my experiments, the combination of Hoechst 33342 and ethidium bromide was used. However, neither exclusion dyes work in postfixed cells. Therefore, the examination by this method had to be rapid. I used the photographic techniques described in chapter 2 for the exclusion of necrotic cells and to study nuclear morphology.

Work and Objectives Summarised

My focus in this thesis is the study of PCD and an evaluation of the Neurotrophic Hypothesis in the murine thalamus. The questions that I addressed include the following. 1) When and where is the period of thalamic PCD? 2) Do any known trophic molecules such as neurotrophins control thalamic PCD? 3) Do target-derived (extrinsic) factors, as compared to intrinsic factors, have influences on thalamic cell survival? 4) Is there any switch in trophic requirement occurring in the developing thalamus and does that match with the target innervation period? 5) Is the transcription factor Pax-6 and/or thalamic innervation essential for thalamic cells to obtain sufficient trophic supply from the target.

The thesis is divided into 6 major experiments (Chapter 2 to Chapter 7).

In Chapter 2, I searched for methods and criteria for studying cell death *in vivo* and *in vitro*. This chapter deals with the regional identification of individual nuclei in the thalamus in histological sections, application of a TUNEL technique *in vivo*, and finding criteria for cell death in cell and tissue culture.

In Chapter 3, I searched for a period of PCD in the thalamus that might correlate with the target innervation period *in vivo*. Moreover, using the *trkB-/-* and *trkC-/-* mice, I studied the role of neurotrophins and their receptors in regulating survival of the developing thalamic cells.

In Chapter 4, I tried to find the specificity of trophic requirement of thalamic cells during the target innervation period and whether there is a switch in trophic requirement by testing different conditioned media obtained from different tissues and ages on thalamic cell cultures.

In Chapter 5, I carried out an *in vitro* investigation of isolated organotypic thalamic explant cultures of *trkB* and *trkC* knockout mice to exclude possible

influences of the target or other features of the external environment that might be involved in compensation for neurotrophin effects.

In Chapter 6, I tested whether the *pax-6* gene is involved in thalamic cell survival. I investigated the possible direct and indirect role of this gene in causing thalamic cell death.

In Chapter 7, I investigated possible role of NT-3 and TrkC signaling on cell proliferation in the brain using BrdU labeling technique.

Chapter 2: *In vivo* and *in vitro* assessment of cell death in pre- and postnatal thalamus

Introduction

Detection of programmed cell death in cells and tissues is important in developmental biology, and was particularly so in my research. Most experiments involved measurement of cell death in thalamic development. Analysis of cell death in the thalamus was the focus of my studies. Numerous methods have been developed for cell death detection. Cytochemical methods have recently been extensively used both in individual cells and in tissues (see review by Willingham, 1999). However, many are not specific for the apoptotic process or PCD. Considerable confusion exists over the interpretation and accuracy of some of these methods and their usefulness in all settings.

Studies of cell death *in vivo* directly observe the actual regressive event occurring in the developing animal. This approach will eventually provide us with a better understanding not only of the role of cell elimination in counterbalancing cell proliferation within the same lineage, but also of the interaction between various cell populations in the same tissue or even those in remote tissues that communicate through paracrine or endocrine loops. To investigate PCD in the *in vivo* physiological condition, some basic requirements for any method of detecting PCD have to be considered. These include 1) resolution at the individual cell level and 2) the requirement that the technique for preserving tissue architecture does not interfere with the detection method (Ben-Sasson et al., 1995). These factors become important particularly in developing tissues of young animals where cells in certain tissues are densely packed. Moreover, cells dying by apoptosis are removed relatively rapidly via phagocytosis (Ben-Sasson et al., 1995). The number of apoptotic cells seen at a particular time point might be low and difficult to quantify by normal nuclear staining. Adequate sensitivity of the selected method is therefore essential.

Nuclear fragmentation labeling by the TUNEL method is commonly used for the detection of apoptosis. It has advantages *in vivo* due to its specificity and selectivity. However, this method still requires care in interpretation, and the details of tissue fixation, permeabilisation, and processing are especially important (Gavrieli et al., 1992). False-positives can be generated (Kockx et al., 1998; Mizoguchi et al., 1998). On the other hand, inaccessibility of tissue sites can prevent the detection of strand breaks (Nakamura et al., 1997). In this study, I tried to optimise the protocol and show that this TUNEL labeling method can be used in the study of apoptosis in the developing thalamus. To obtain a more accurate TUNEL quantification, a fluorescent technique was used instead of the normal enzymatic color reaction. Some advantages of using fluorescent methods over the conventional colorimetric TUNEL technique are: 1) the advantages for measurement, since counterstaining with fluorescent nuclear dyes provides better resolution for cell counting (high sensitivity and low background); 2) it can be useful for multiple immunohistochemistry detection.

Before I started to verify the TUNEL method in tissue sections, I needed to be able to accurately identify the regions of the thalamus. This was because the fluorescent counterstains that I used stained only nuclei, and the fiber tracts and other cellular components normally seen in Nissl or haematoxylin stainings were not visible with fluorescence microscopy. Thus the identification of different brain structures was not as clear as with normal Nissl or haematoxylin stained sections, and there was a danger that thus would result in an erroneous interpretation of the location in which PCD takes place.

For *in vitro* studies, on the other hand, the problems associated with measuring cell death are different. Lower cellular densities in cultures allow us to observe dead cells easily and cell remnants remain undisturbed since there is no cell clearance by macrophages (Johnson, 1995). Cell culture is more accessible to study the cellular detail of dying or dead cells. However, the isolated cells have to regenerate and adapt to their new culture environment. Cells in culture may therefore be selected for either survival or cell death by events that do not correspond to those

occurring in the normal developing embryo (Johnson, 1995). Disruption of cells during certain preparations such as tissue dissection and dissociation can cause injury and lead to necrosis. The criteria for identification of cell death *in vitro* are thus essential. This sometimes makes the tissue culture approach to cell death as difficult as a study *in vivo* (Johnson, 1995; Willingham, 1999).

Here I tried to set criteria for detecting apoptosis and other mode(s) of cell death (i.e. necrosis) *in vitro*. There are several methods for detection of apoptosis *in vitro*. For example, we can analyse the sequence of morphological changes during apoptosis *in vitro* by using time-lapse microscopy, but this technique is better suited to monitoring small groups of cells than for quantification of large populations (Willingham, 1999). Nevertheless, following the morphological appearance with time lapse observation can provide some criteria by which to determine viability and death of cells in culture. These criteria include 1) cell size, which will be greatly reduced in the late stage of apoptosis (Willingham, 1999), 2) cell brightness under phase contrast microscopy (dead cells are often phase-bright) (Lotto, 1994; Davies, 1996), 3) certain neuronal growth indicators, such as the measurement of neurite outgrowth, which have also been suggested to correlate with cell viability (i.e. it has been suggested that apoptotic cells or dead cells usually lack neurites) (Baserga, 1995; Lotto, 1994; Lotto et al., 1997). Whether or not any of these criteria alone is sufficient to identify cell death or is correlated with a particular type of cell death is still unclear.

In order to distinguish necrosis from apoptosis, I used a fluorochrome combination technique (a nuclear dye, Hoechst, and an impermeable dye, ethidium bromide – EB) to stain live cultures (see also Chapter 1). With this technique, cells can be sorted into 4 groups: (a) viable cells with normal nuclei stained blue (red EB excluded); (b) necrotic cells stained red (EB can enter) with normally appearing (either larger or normal sized) nuclear structure; (c) early apoptotic cells with blue pyknotic nuclei (red EB excluded); and (d) late apoptotic cells with red pyknotic nuclei (Coligan et al., 1995).

With regard to the analysis of nuclear morphology by DNA-binding dyes, nuclear condensation changes seen with these dyes *in vitro* are easier to follow than *in vivo*. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by these fluorochromes. Moreover, Dive et al. (1992) have also reported that with Hoechst staining, apoptotic cells have stronger blue fluorescence compared to non apoptotic cells, but one disadvantage of this method is the variability of Hoechst uptake in different cells and its possible change during certain treatments. Therefore, the ability to discriminate apoptotic cells from normal cells by observing increased uptake of dye has to be tested for each new cell system (Schmid et al., 1994). Some fluorochromes (acridine orange (AO), SYTO-13, and Hoechst 33342) were tested here to see whether they stain nuclei differently. However, the method has the drawback that it is a subjective measurement, i.e. there is no clear cut-off point between normal and apoptotic cells. Since clear morphologically distinct apoptotic nuclei appear late during apoptosis, this may lead to an underestimation of apoptotic cell number (Wyllie et al., 1998). Based on my own observations, I identified a novel group of cells with 'intermediate nuclear condensation'. This was a population of cells that had gross nuclear morphology similar to that of normal viable cells but with some increase in the density of condensation or in the number of chromatin clumps together with, in many cases, increased intensity of Hoechst staining (as occurs in apoptotic cells) (see Results; Wyllie, 1998). The morphology of this group of cells also resembles that described previously as displaying early nuclear change in apoptosis (Kerr et al., 1995). I followed the change in percentage of this population in two *in vitro* experimental sets in this chapter.

In the first set of experiments E15 thalamic cells were plated at various densities (500, 1000, and 3000 cells/mm^2) to 1) observe and correlate changes in size, phase brightness, and production of neurites of cells which might help in determining cell viability; 2) estimate the numbers of necrotic cells in cultures; 3) identify and categorise the cells showing intermediate nuclear condensation, as to whether they were healthy, or dying by necrosis or apoptosis. Following the results of the observations on nuclear changes, I questioned whether cells with intermediate

nuclear change morphology are actually dying, by monitoring nuclear changes in high-density thalamic cells over a period of 2 days in cultures.

In summary, this chapter deals mostly with the problems encountered in setting up methods for determining and quantifying cell viability and apoptosis *in vitro* and *in vivo*.

Materials and Methods

Animal/Tissue preparation

C3H mice were obtained from overnight mating, and the day of the vaginal plug was deemed embryonic day1 (E1). Plugged females were separated from the male. Pregnant mice aged E13 to P1 were killed by cervical dislocation. Embryos were removed by Caesarean section and decapitated. The brains were then removed. Only E15 brains were used for tissue culture (for methods, see below). For tissue embedding, the brains were fixed with 4% parafomaldehyde in phosphate buffered saline (PBS) overnight, washed several times with PBS, dchydrated and paraffin embedded as in standard histological protocols. Coronal sections were cut at 7μm and mounted on 0.01% poly-$_L$-lysine coated slides. One in every five sections was mounted on the same slide to make sections alternate.

Tissue Cultures

For tissue culture, dorsal thalamic explants were dissected out in oxygenated Earl's balance salt solution (EBSS) as described in Lotto and Price (1995). They were dissociated enzymatically for 45 minutes at 37°C in EBSS containing papain (10 units/ml) (Worthington Biochemical Dissociation Kit). Then the tissues were triturated 8-15 times with a flame-polished (to obtain rounded smooth end) pasteur pipette. When the explants had become cell suspensions in EBSS, they were centrifuged at 300g for 5 minutes at room temperature. The pellet was resuspended with 1:1:10 albumin-ovomucoid inhibitor solution, DNase solution, and EBSS respectively. The resuspended cells were layered on top of the high density ovomucoid inhibitor and were centrifuged at 70g for 6 minutes. The pellet of dissociated cells was resuspended in 1ml culture medium. This volume is termed as $V_{\text{cell suspension}}$, would be used for calculation of plating density (see below). The cell suspension was further diluted to the desired plating density and plated in 96 well-plates (Costar) which were pre-coated with poly-$_{DL}$-ornithine or poly-$_L$-lysine solution (Sigma) (see below). Once plated, the cultured cells in the well were

incubated in an O_2:CO_2 incubator at 37°C until the period of culture was finished. Then cells were fixed with 4% paraformaldehyded in PBS for 15 minutes, and washed 3 times with PBS. Nuclear marker fluorochromes were applied 15 minutes either before (dyes added to culture medium) or after (dyes added to PBS) the fixation.

In this study, E15 thalamus was used in dissociation studies because:
1) Conditions for culture of this tissue have been optimised (Lotto, et al., 1997).
2) The majority of the cells is neuronal (Lotto, 1994).
3) Cells survive well after 24-48h, with no apparent dependency on exogenous trophic factors.

Identification of thalamic nuclei

Thalamic nuclei and boundaries were identified according to the Atlas of Prenatal Mouse Brain (Schambra et al., 1992) and the Atlas of Developing Rat Brain (Paxinos et al., 1994). Dorsal thalamus was identified as a mass of cells between the external medullary lamina (eml) (from ventral thalamus) and fasciculus retroflexus (fr – hablenulo-peducular tract) from the dorsal thalamus.

TUNEL

The TUNEL protocol on sections was performed with some modifications from the original protocol described in Gavrieli et al., (1992). The peroxidase technique was replaced with a fluorescent technique. After routine rehydration, tissue slides were treated with 20µg/ml proteinase K, further fixed for 10 minutes with 4% paraformaldehyde, and then treated with 1% Triton-X in PBS for 5 minutes. 70-100µl of TdT (terminal deoxynucleotidyl transferase) mixture was applied to each slide and incubated for 1½h in 37°C.

These TdT mixtures comprise TdT enzyme (Promega, final concentration 30 units/ml) and 50nM biotinylated-dUTP (Boehringer) in TdT buffer (30mM Tris pH7.2, 140mM sodium cacodylate, 1mM $CoCl_2$).

The reaction was stopped by SSC washing. Slides were next washed with PBS and then incubated with 1:200 fluorescein conjugate, Streptavidin-Alexa 488 (Molecular Probes). Tissues were finally washed by PBS, mounted and counterstained by either Vectashield-DAPI or Vectashield-propidium iodide (Vector). Sections pretreated with 10ug/ml DNaseI served as positive controls for all of the subsequent experiments (also for ones in Chapter 3, and in Chapter 6). For negative controls, the TdT enzyme was omitted.

The method for TUNEL in cell culture was similar to that in slides. After cells had been fixed and washed 3 times with PBS, 60μl TdT mixture was added to each well. All washes were the same as for sections. Finally the cells were counterstained with 1mg/ml propidium iodide (PI).

Calculation for plating density

10μl of cell suspension in culture medium was placed on a haemocytometer. I calculated the density of cells by counting number of cells in 5 x 5 grid square of the haemocytometer, where the large square (25 mini-squares) is covered by approximately 100nl. When multiplied by 10, this gives the number of cells per μl.

To obtain the volume to make a correct plating density, the dilution factor was determined. The calculation for the dilution factor was as follow.

$$\text{Dilution factor} = \frac{(\text{number of cells}/\mu\text{l}) \times (\text{volume to be add per well, } \mu\text{l})}{(\text{area of a culture well, mm}^2) \times (\text{plating density, cells/mm}^2)}$$

The total volume (V_{total}) of culture medium needed for the given density of cell suspension to make a determined plating density will equal the volume of the cell suspension times the dilution factor.

i.e. V_{total} (ml) = $V_{cell\ suspension}$ (ml) x dilution factor.

The volume of culture medium (V_{add}) to be added to the to the cell suspension will be:

$$V_{add}\ (ml) = V_{total}\ (ml) - V_{cell\ suspension\ (approximatly\ 1ml)}$$

Cells were plated at low (500 cells/mm^2), medium (1000 cells/mm^2), or high (3000 cells/mm^2) densities. Cells were incubated for 24 hour for plating density experiments.

For experiments to test the effects of culture period, I plated only at high density (3000 cells/mm^2). Cells were cultured for 2h, 24h, or 48h.

Fluorescence staining for cell cultures

Initially, several fluorochromes were applied to cells, either alone or in combinations. These dyes included acridine orange (AO, green in a fluorescence filter), Hoechst (HO, seen with a UV filter), SYTO-13 (green in a fluorescence filter), propidium iodide (PI, red in a rhodamine filter), and/or ethidium bromide (EB) (red/orange in a rhodamine filter). Nevertheless, later findings showed that AO, HO, and SYTO-13 stained nuclear similarly, while PI was similar to EB. Thus, HO was chosen for single staining while HO and EB were chosen for combination staining. In experiments varying plating densities, the dyes (10-20μg/ml) were dissolved in fresh medium, applied to unfixed cells (to retain the membrane integrity of live cells, which would exclude EB) and photomicrographed. In subsequent experiments, only 10μg/ml Hoechst in PBS was directly applied to fixed samples.

Measurements

Cells were classified and described by either one or more of the following criteria; 1) cell size (large cells or small cells relative to neighbouring cells), 2) phase-brightness under phase-contrast microscope (phase-dark cells or phase-bright cells), 3) neurite production (neurite-producing cells – cells with the length of a

process which should be at least equal to or longer than the diameter of the cell body, or cells without neurites), 4) fluorescent dye uptake (blue cells – Hoechst positive, or red/orange cells – EB positive), 5) nuclear morphology (either pyknotic cells or non-pyknotic cells). In subsequent experiments, cells which were not pyknotic were subdivided into cells with diffuse chromatin and cells with intermediate chromatin condensation.

Healthy cells are cells that are relatively large, phase-dark, produce neurites, have diffuse chromatin, and are Hoechst-positive. In some cases (especially in the later experiments) the intermediate group was considered to comprise normal cells (see Results).

Numbers of experiments and counting details

1. For *in vivo* experiments, four embryos were used for identification of thalamic nuclei and TUNEL. One adult mouse was used as a control for TUNEL.
2. For *in vitro* experiments, around 8 mice were used for both plating density experiments and 2-day experiments for monitoring nuclear changes. Each group contained at least 5 wells (n = 5). For each well, cells were counted in three randomly selected areas of $500\mu m^2$.

Results

1. Anatomy of the thalamus viewed following Hoechst staining

At E13, dorsal thalamus was differentiated from the ventral thalamus by a cell-sparse horizontal band called the zona limitans intrathalamica, and from the epithalamus by another thin cell-sparse band called the p1/p2 border. In Hoechst stained sections, these two bands were seen as strips of low nuclear staining. The subdivision into thalamic nuclei could not be defined at this age. At E15, some thalamic nuclei could be seen e.g. paraventricular nucleus (pvt), dorsal lateral geniculate nucleus (dlg) and posterior nucleus. However, the many subdivisions into other thalamic nuclei, particularly ventral nuclei, were difficult to determine and indistinct. Classification of these unclear-bordered thalamic nuclei was as groups of thalamic nuclei.

From E17 onwards, all major thalamic nuclei were discernible with Hoechst staining. The thalamic nuclei are shown in Figure 2.1. In column II - b, d, f, h, and j show E19 thalamus stained with Hoechst. The thalamic nuclear subdivisions were visible and were identified in a similar way as under Nissl staining (Figure 2.1 column I – a, c, e, g, and i, taken from Schambra et al, 1992). However, the intensity of the staining with Hoechst was relatively more homogeneous, while in Nissl stains there was better contrast among structures in the tissue. This made the identification of thalamic nuclei slightly more difficult with Hoechst but some other criteria such as shape, cell density, and position were used to help identify individual thalamic nuclei. At the anterior part of the thalamus (Figure 2.1a, b), paraventricular nuclei, the anterior part of the reticular nuclei, and the anterior nuclei were firstly seen. The paraventricular nucleus in Figure 2.1, with lobulated shape, lies along the 3rd ventricle and medial to a large bundle of fibers of the stria medularis. The reticular nucleus, in the same section, lies horizontally underneath the zona limitans intrathalamica.

Figure 2.1

Pictures illustrating the anatomy of the thalamus and its subdivision into the thalamic nuclei in coronal view, according to the Atlas of Prenatal Mouse Brain (Schambra et al., 1992) (Column I). Sample of thalamic tissue at around E19, stained with DAPI, shown in Column II. The boundaries of each thalamic nucleus are dotted lines.

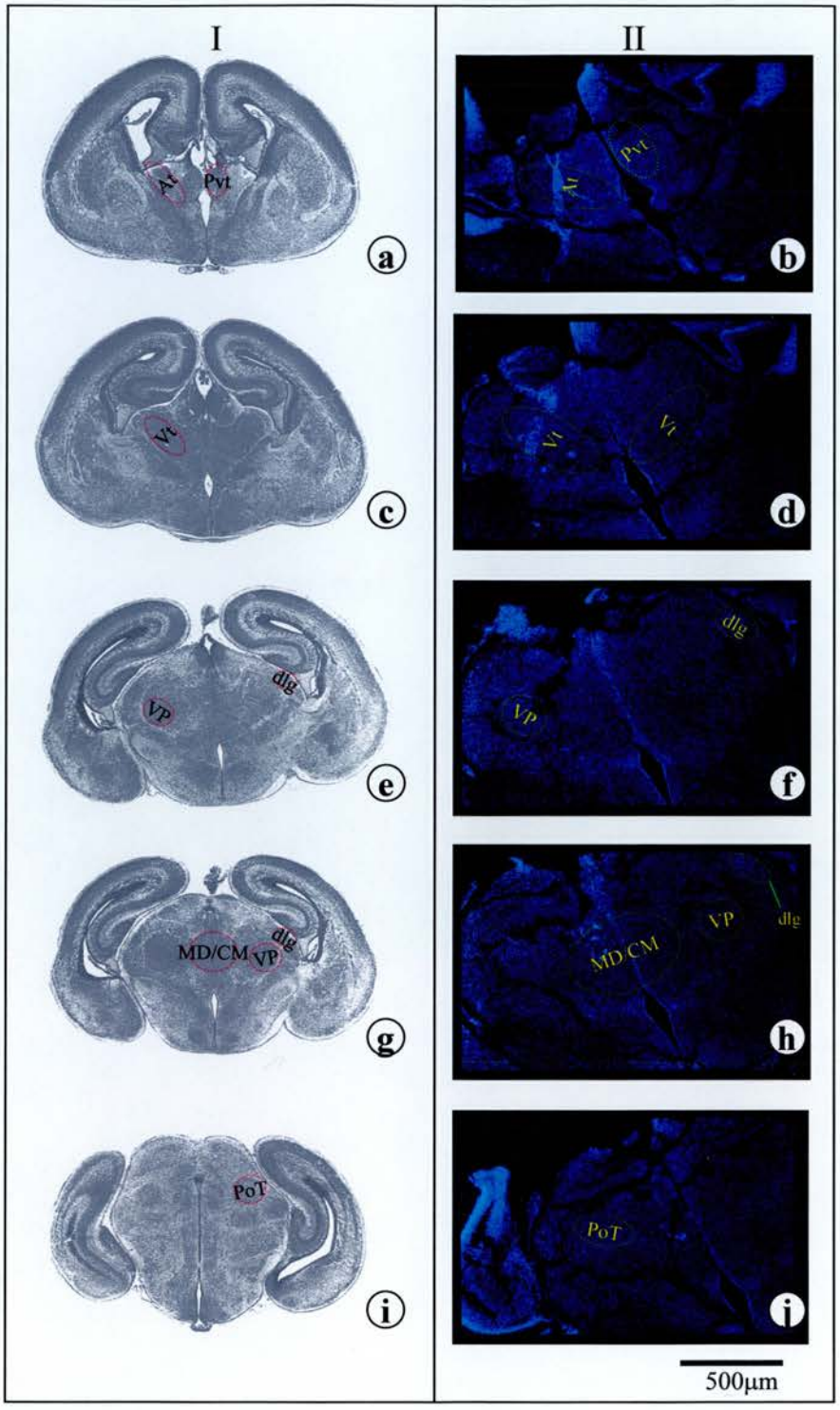
(a) and (b) Anterior-most section of the thalamus showing the area of the paraventricular nucleus (Pvt) and anterior thalamic nuclei (At).

(c) and (d) Section showing ventrolateral and ventromedial thalamic nuclei (VL/VM, Here I called Vt).

(e) and (f) Section showing ventroposterior lateral and ventroposterior medial thalamic nuclei (VPL/VPM, Here I called VP). Mediodorsal and centromedial thalamic nuclei (MD/CM) are also shown as well as dorsal geniculate nucleus (dlg).

(g) and (h) VP (VPL/VPM) in a more posterior section. MD/CM and dlg are still present and posterior thalamic nucleus (PoT) is first to appear.

(i) and (j) Area of the posterior thalamic nucleus (PoT) that is used for cell counting.


I
II
Avt
Pvt
a
b
Vl
c
d
VP
dlg
e
f
MD/CM
g
h
PoT
i
500μm

In a more posterior section, as in Figure 2.1c, d, the posterior part of the anterior nuclei was found lateral to the stria medularis and was dorsal to the ventricular nuclei and the reticular nucleus. Ventral group nuclei lay lateral to the intralaminar nuclear group and dorsal to the reticular nucleus. Cells in these nuclei were loosely packed.

In Figure 2.1e, f, the ventral posterior nuclei can be easily defined by their lobulated appearance. However, in a very posterior part of the nuclei, it was difficult to find a clear border between these nuclei and the medial geniculate nucleus at this age (E19). It has been reported previously that medial geniculate nucleus may merge with the ventral posterior nuclei in adults of small species (Jones, 1985). Dorsal geniculate nuclei were distinct due to their clear borders and bright staining of the densely packed cellular nuclei (Figure 2.1e-h).

Intralaminar, centromedial, and mediodorsal nuclei lie on the inner side of the thalamus covered by the ventral, ventral posterior, and posterior nuclei. They lie along the ventricle and may contact the other side of the thalamus (Figure. 2.1g, h). The rostral part of the posterior nucleus lies dorsally to the ventral posterior complex. However, the boundaries of the posterior nucleus were less clear at the caudal end (Jones, 1985) but it can be roughly identified by a loosely packed cell component, which lies ventro-lateral to the parafascicular tract and medial to the geniculate bodies at the posterior end of the thalamus.

More figures of thalamic nuclear divisions can be seen in the photomicrographs of the thalamus with *trkB* and *trkC in situ* hybridisations in Chapter 3 (Figure 3.1).

2. TUNEL labeling in thalamic sections.

Firstly, I verified the TUNEL technique by examining the reacted sections of adult brain (cerebral cortex) 1) with the omission of the TdT enzyme during the incubation as a negative control (Figure 2.2a-c), and 2) treated with DNaseI as a positive control (Figure 2.2d, e). Adult mouse brain was used here because apoptosis

Figure 2.2

Photomicrographs showing positive and negative controls used for TUNEL reactions.

(a) Negative control: Coronal section of an adult cerebral cortex with TdT omitted and stained with propidium iodide (PI, red color in (b)). No cells are labelled. However, autofluorescent cells can be observed (yellow cells indicated by white arrows).

(b) Same section as (a) counterstained with propidium iodide (PI) under the rhodamine filter. All cells are non-specifically stained.

(c) Same section as (a) under UV filter. Tissue is not stained with any dye for this filter but autofluorescent signals (white arrows) are very strong.

(d) Adult positive control: Coronal section of an adult cerebral cortex pretreated with DNaseI and stained with propidium iodide (PI, red color in (b)). Autofluorescent artifacts (white arrows) appeared more yellow than DNase-induced positive cells (which appeared green, red arrows).

(e) Same section as (d) counterstained with propidium iodide (PI) under rhodamine filter.

(f) E19 positive control: Coronal section of an embryonic thalamus treated with DNase to fragmentate DNA. All cells were stained with TUNEL reaction (green).

(g) Same section as (f) counterstained with DAPI under UV filter. All cells are stained.

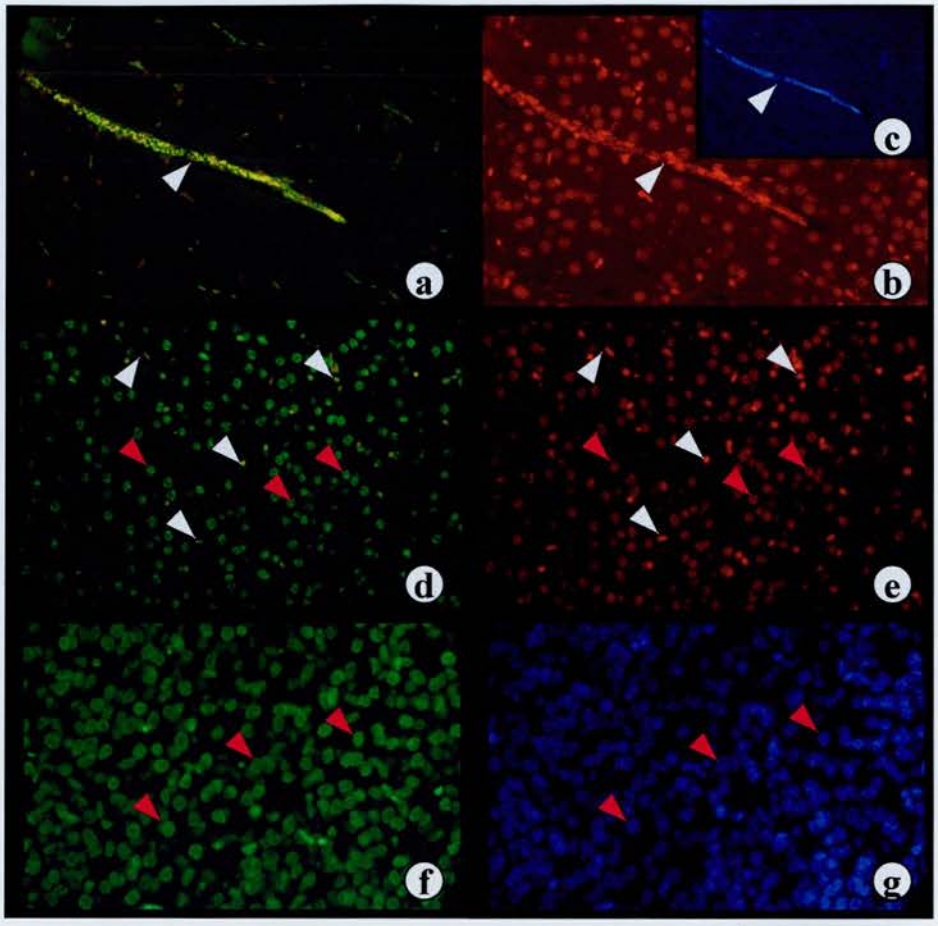

a
b
c
d
e
f
g
100μm

Figure 2.3

Photomicrographs showing examples of TUNEL positive cells *in vivo* in experimental E19 brain section and *in vitro* in thalamic cultures.

(a) Fluorescent TUNEL reaction detects specifically pyknotic (apoptotic) cells (green) *in vivo*. Section obtained from E19 posterior thalamic nucleus.

(b) Higher magnification of a TUNEL positive cell (from dotted box line in (a)) showing pyknotic morphology. Note: no adjacent cells were picked up by the TUNEL reaction.

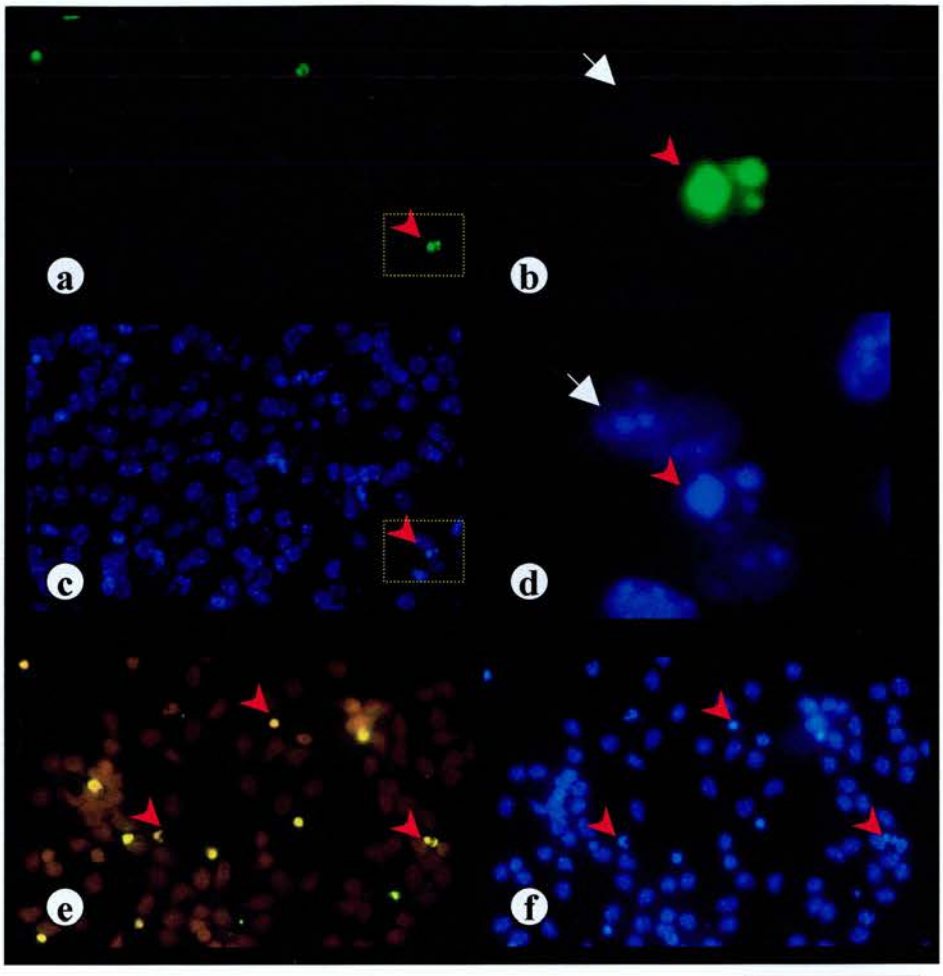
(c) Same section as (a) of tissue viewed under UV filter. DAPI non-selectively stains all nuclei of cells but the pyknotic (presumably apoptotic) cells are brighter stained (as seen in (d)).

(d) DAPI staining of the same position as in (b) (from dotted box line in (c)) viewed in UV fluorescence at higher magnification.

(e) TUNEL reaction in culture counter-stained with propidium iodide (PI) and Hoechst. The TUNEL positive cells appeared yellow. This may be due to high concentration of PI used (20 μg/ml). No autofluorescent cell was found.

(f) Hoechst staining of the same position as (e) viewed in UV fluorescence.

Red arrows depict TUNEL positive cells (all are pyknotic) while white arrows are TUNEL negative cells.



100μm (a,c,d,f)

20μm (b,d)

in adult brain is known to be very low (Blaschke et al., 1996). In positive controls, DNaseI caused DNA fragmentation like that in apoptosis. Any TUNEL labeling in the positive control should come mostly from the DNaseI digestion. Virtually all cells treated with DNaseI, including cells that do not show pyknotic morphology, had their DNA broken and were labeled green with fluorescent TUNEL (Figure 2.2d, e). Some yellowish signal was seen in certain cells but was due to endogenous autofluorescence, which may quench TUNEL. This was only found in blood vessels (blood derived cells/endothelium), and in the pial region. Contrary to the true TUNEL labeling, autofluorescence was non-specific and could be seen also with rhodamine and UV filters. These autofluorescent objects were also seen in the negative control in the adult cortex even though the TdT enzyme was omitted (Figure 2.2a-c).

In Figure 2.2f, g, I further confirmed that TUNEL could be used in the embryonic tissue (E19 thalamus), with the treatment with DNaseI as positive control. Moreover, in the experimental sample, when E19 thalamus (posterior area) was reacted with the TUNEL reaction, only cells showing apoptotic figures (chromatin condensation and fragmentation) were labeled (Figure 2.3a-d). This suggested that TUNEL was specific to apoptosis. Although only pyknotic cells were labeled, the labeled cells appeared more yellowish in culture than *in vivo* (Figure 2.3e, f). The change in color may have been due to some difference in the fixing method or to the high-concentration of the PI counterstain.

3. Thalamic cells in culture

3.1 Cell morphology and viability in different plating densities

Once plated, most cells were round, without neurites and phase dark but their cell size was variable. This variety in cell size may reflect different cell types/subpopulations existing in the developing thalamus *in vivo* (Jones, 1985), and does not necessary reflect viability of cells *in vitro*. Thus, the use of cell size as a criterion for assessing cell viability would not be simple and I did not use this

Figure 2.4

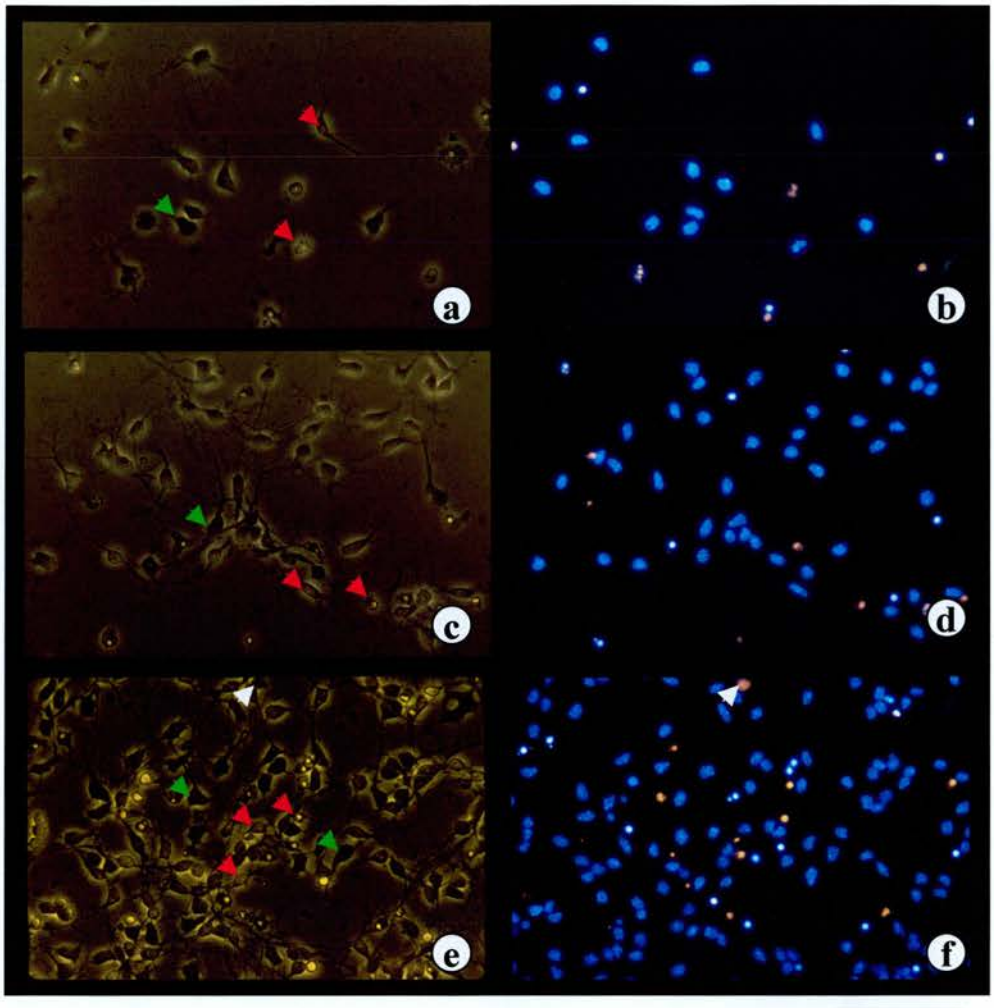
Photomicrographs of cultured thalamic cells at different plating densities.

(a) and (b): Phase contrast and fluorescence microscopic views of thalamic cells (stained with Hoechst and ethidium bromide) at 500 cells per mm^2 plating density. (a) Phase bright cells are seen (red arrows). Cells either have short processes or do not have any at all. (b) Some pyknotic cells with either ethidium bromide (orange) or Hoechst (blue) are clearly seen.

(c) and (d) Phase contrast and fluorescent microscopic views of thalamic cells at 1000 cells per mm^2 plating density. (c) More cells produce neurites and start to make contacts. Some phase bright cells are shown with arrows. (d) The proportions of pyknotic nuclei are reduced.

(e) and (f) Phase contrast and fluorescence microscopic views of thalamic cells at 3000 cells per mm^2 plating density. (e) Most cells are phase dark and produce neurites. (f) A higher proportion of live cells are seen, stained with Hoechst with diffuse chromatin in their nuclei.

Green arrows; phase-dark cells, red arrows; phase-bright cells, necrotic cells; white arrows.


a
b
c
d
e
f
100μm

criterion further. Thalamic cells in 24h cultures had different sizes, phase brightnesses, extents of neurite production and fluorescent dye uptakes. Cells with these appearances were found in all plating densities but in different proportions (Figure 2.4, finer detail of nuclear morphology and dye uptake classification are in Figure 2.5). Most of the plated cells that produce neurites are multipolar cells. Extensive production of neurites can be seen at this period. These cells have at least one thick neurite, which is an axon-like process and sometimes has a growth cone. These multipolar cells in cultures are morphologically similar to those thalamic neurons found *in vivo*.

a) Cell brightness under phase contrast microscopy

The proportions of phase bright cells were different at different plating densities (Figure 2.6, row 1). 59% of thalamic cells at low density (500 cells/mm^2, n = 5) were phase dark while 66% of cells in high density (3000 cells/mm^2, n = 5) were phase dark. However, there were only 54% of phase dark cells at 1000 cells/mm^2 plating density (n = 5) (which is even lower than the percentage at low density). Thus, it was not clear that the percentages of phase dark cells were directly propportional to the increase in plating density. Moreover, although most dead cells (cells with pyknotic morphology, see below) appeared to be phase-bright, there were some phase bright cells which were healthy-looking, with neurites.

b) Production of neurites

As the plating density was increased, markedly more cells produced neurites. 60%, 78% and 95% of cells produced neurites at plating densities of 500 cells/mm^2, 1000 cells/mm^2, and 3000 cells/mm^2 (n = 5 each) respectively (Figure 2.4, 2.6, row 3). This suggested that an increase in cell density *in vitro* would support more cells to either grow or survive or both. Although this growth of neurites seemed to be related with the survival of thalamic neurons, it is not known whether this change (production of neurites) is a direct effect of the increase in the viability or whether there is some other separate mechanisms controlling this growth. Some dead cells

may not have fully retracted their neurites before death, and some viable cells may not produce neurites.

c) Nuclear morphology of thalamic cells *in vitro* as seen by several nuclear fluorochromes

Several dyes such as acridine orange (AO), SYTO-13, Hoechst, DAPI, PI and EB were tested. As previously described, both PI and EB stain all nuclei in fixed tissue, but in living tissue PI and EB only enter damaged cells so they are not much use for vital staining of nuclear morphology. I have tested all these dyes and found no apparent difference among them in their ability to reveal nuclear morphology. Thus only one dye was chosen for subsequent studies of nuclear structure. In the case of cell culture I used Hoechst, while EB was used mainly as a membrane exclution dye in the combination staining.

The initial analysis of nuclear morphology revealed two major populations of cells: non-pyknotic and pyknotic cells. Percentages of non-pyknotic cells increased continuously (65%-74%-80%) when the plating density was increased from low plating densities of 500 cells/mm^2, to 1000 cells/mm^2 and high density, 3000 cells/mm^2 respectively (Figure 2.6, row 3). However, this method could not differentiate necrotic populations (some of which are non-pyknotic). Moreover, there was a problem in identifying some cells, which had an intermediate morphology in between pyknotic and non-pyknotic cells. This was because there was no clear cut-off point between normal and apoptotic cells.

d) Nuclear morphological analysis with a new 3-fold classification

When cultured cells were classified into three categories – cells with diffuse chromatin (Figure 2.5a, b, and f), cells with intermediate condensed chromatin (intermediate cells) (Figure 2.5c, d, e), and pyknotic cells (Figure 2.5g, h), percentages of cells with diffuse normal chromatin increased in proportion to the rise of plating density (from 22%-26%-41%, n = 5 each) (Figure 2.6, row 4). The ratios of pyknotic cells also decreased gradually as the plating density was increased.

Figure 2.5

Photomicrographs showing thalamic nuclei with different nuclear morphology, double-stained with Hoechst and ethidium bromide (enlarged from selected cells in Figure 2.3c).

Group 1: (a)-(e), this group can be subdivided into two other broad categories.

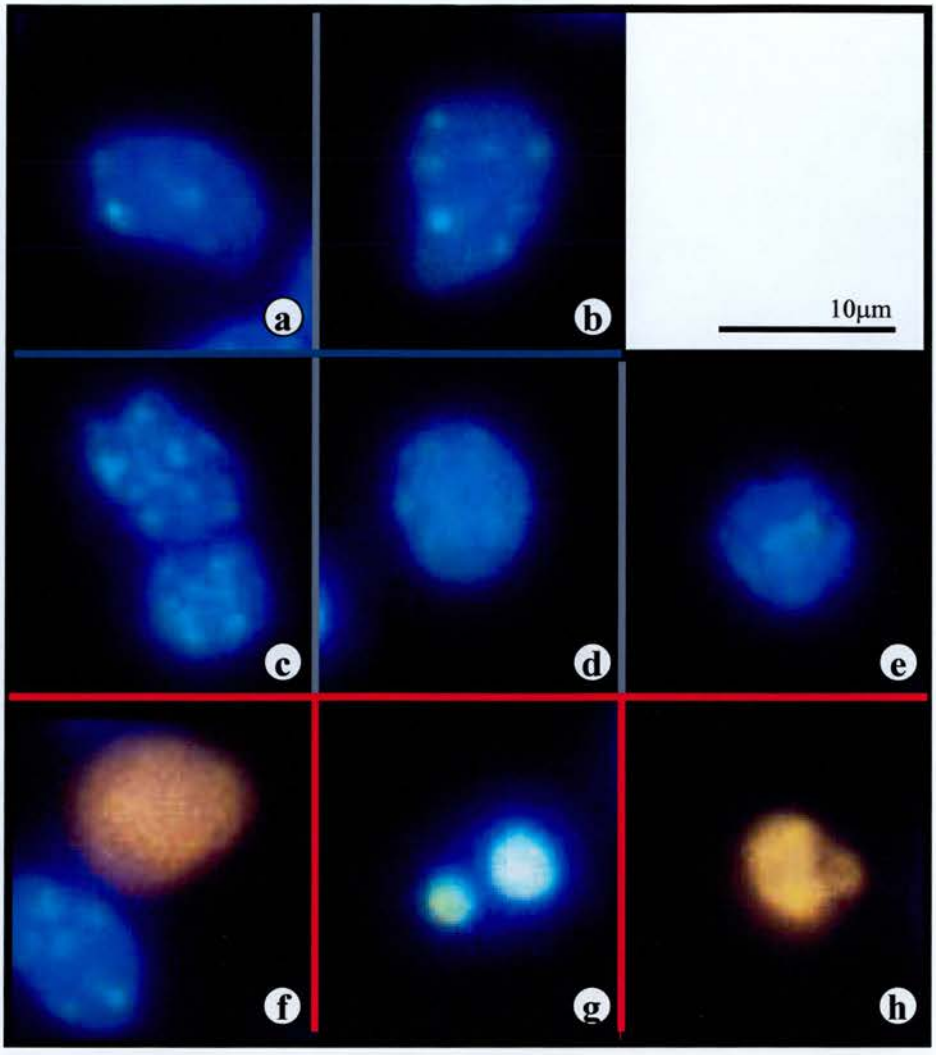
1.1) (a) and (b) are examples of cells with diffuse chromatin.

1.2) (c), (d) and (e) are cells of intermediate nuclear morphology ((c), cells with clumps of chromatin; (d), cell with bright homogenous Hoechst staining, and (e), small cell with unclear nuclear structure).

Group 2: (f) necrotic cell lets ethidium bromide enter the nucleus and is stained orange.

Group 3: (g) early-apoptotic cell is stained blue with pyknotic nucleus.

Group 4: (h) late-apoptotic cell is stained orange (due to the increase permeability to ethidium bromide) with pyknotic nucleus.


a
b
10μm
c
d
e
f
g
h

However, the change in percentage of intermediate cells was discontinuous when plating density was increased (43%, 48%, and 39% at 500, 1000, and 3000 cells/mm^2 plating densities accordingly)(Figure 2.6 row 4). This led to the speculation that the intermediate cells may be either cells in an intermediate state that were preparing to die (either by necrosis or apoptosis) or cells in other cellular state(s) of metabolism which were not dying. I attempted to further investigate this by monitoring changes in proportions of these cells over a period of 2 days in culture (see below).

e) Fluorochromes for combination staining

With a combination of Hoechst 33342 (blue) and ethidium bromide (EB, red), dead cells with comprimised membranes were clearly visible by red staining with EB. The percentages of these cells and Hoechst stained cells at different plating densities are shown in Figure 2.6, row 5. High-density cultures had lower proportions of EB positive cells. This method could not differentiate early apoptotic cells and necrotic cells and thus was less useful without combining it with the nuclear morphological analysis. When analysing the cell color with an appearance of pyknosis, percentages of cells with non-pyknotic nuclei with blue color were ranging from 63% to 80% (n = 5 each), from low- to high- plating densities. The proportion of non-pyknotic cells stained with EB (which represents the number of necrotic cells) is very low (< 1%). Pyknotic cells with blue color were ranging from 19.07%, 14.24%, to 12.94% (from 500 to 3000 cells/mm^2, n = 5 each), while late apoptotic cells (pyknotic with red/orange color) were decreased from 16.14% to 7.93% when the density was increased.

f) Correlation among different morphological criteria in determination of cell viability

Since the proportion of necrotic cells was very low under all conditions and could be disregarded, the analysis of combination staining was no longer essential for subsequent experiments (as I could determine nuclear morphology by Hoechst alone). Three criteria were considered in this analysis including phase brightness, neurite production, and pyknotic morphology (the analysis of the intermediate cells

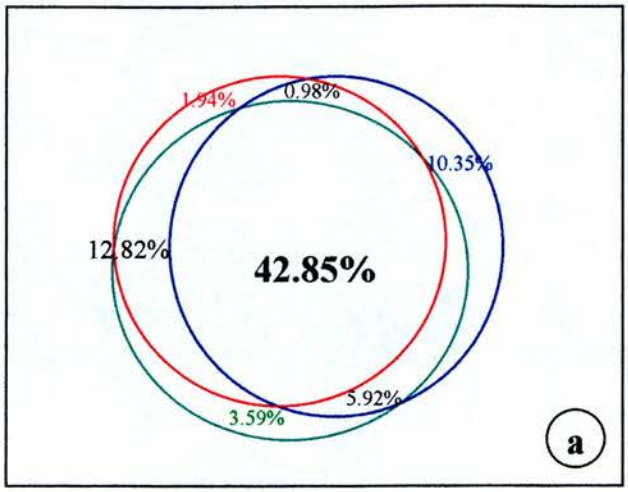
Figure 2.6

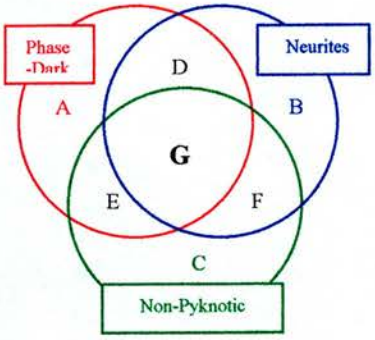
Diagrams showing the percentages of different types of cells in cultures sorted by different criteria at different plating densities. Each plating density (500 cells/mm^2, 1000 cells/mm^2 and 3000 cells/mm^2) is shown in column I, II and III. Each category is sorted in rows 1-5: row 1 = cells sorted by phase brightness, row 2 = cells sorted by the production of neurites, row 3 = cells sorted by pyknotic morphology into either pyknotic or non-pyknotic (when differentiate cells with intermediate nuclear morphology from the diffuse ones, the proportions of cells were shown in row 4), and row 5 = cells sorted by the fluorescent dye exclusion. Red areas indicate proportion of dead or dying-like cells (in percentages) while blue areas might all be healthy looking cells.

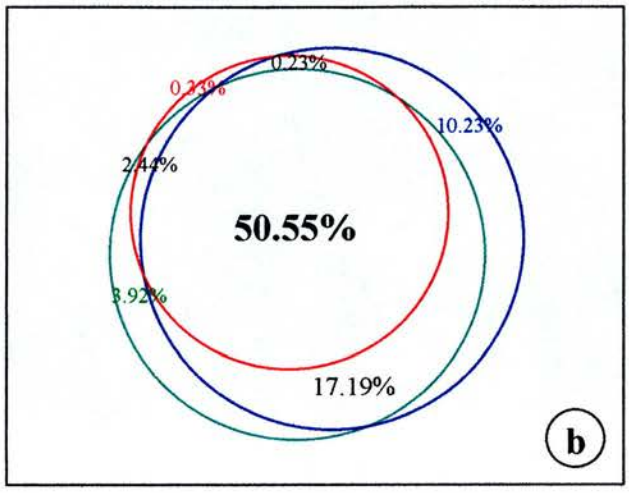
I. (500 cells/mm^2) II. (1000 cells/mm^2) III. (3000 cells/mm^2)

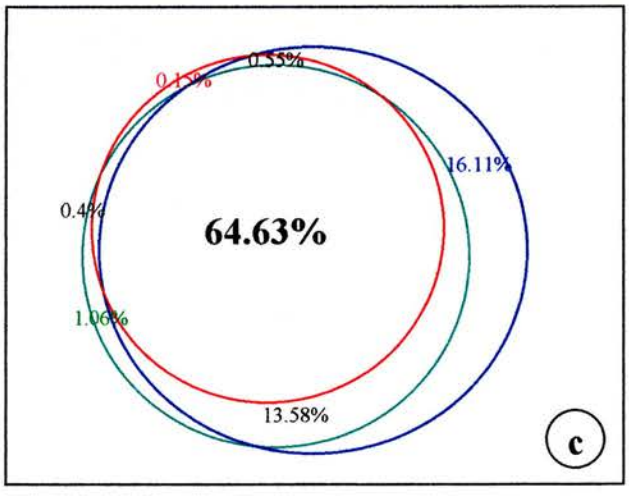
Figure 2.7

Set diagrams showing the proportions of different types of cells. Diagrams of thalamic cells at plating densities of 500 cells/mm^2, 1000 cells/mm^2 and 3000 cells/mm^2 are shown in (a), (b) and (c) respectively. Phase-dark cells are within a red circle, cells producing neurites are within a blue circle, and non-pyknotic cells are within a green circle. Areas A-G in (a)-(c) are proportions of (A) pyknotic phase-dark cells without neurites, (B) pyknotic phase-bright cells producing neurites, (C) non-pyknotic phase-bright cells without neurites, (D) pyknotic phase-dark cells without neurites, (E) non-pyknotic phase-dark cells without neurites, (F) non-pyknotic phase-birght cells producing neurites, and (G) non-pyknotic, phase-dark cells producing neurites. Proportion of cells in each category is shown as average percentages (per total number of cells counted in each plating density). For example area E of (a) shows that 12.82% of cells in low plating density culture are phase dark and non-pyknotic, but do not produce neurites. The rest of the percentages not shown here are proportions of dead cells. Note: many healthy-looking cell share overlapping criteria in area G.


1.94%
0.98%
10.35%
12.82%
42.85%
5.92%
3.59%
a


Phase
-Dark
Neurites
A
D
B
G
E
F
C
Non-Pyknotic


0.23%
0.23%
10.23%
2.44%
50.55%
3.92%
17.19%
b


0.55%
0.15%
16.11%
0.4%
64.63%
1.06%
13.58%
c

was not completed at the time of this present analysis, and I classified nuclei of cells only into 2 types – non-pyknotic and pyknotic cells).

Populations of thalamic cells after culture at different plating densities were displayed in set diagrams, as shown in Figure 2.7a, b, and c, given that the total population of cells in each plating density in the square equals 100%, red circles represented the proportion of phase dark cells, blue circles represented cells with neurites and green circles represent non-pyknotic cells. Overlapping areas (D-G) represented the proportions of cells that share those appearances (e.g. in area G of a set at 500 cells/mm^2 in Figure 2.6a, there was an average of 42.85% of cells which were phase-dark, non-pyknotic and produced neurites). The rest of the cells outside the circles were phase-bright, pyknotic cells without neurites.

A major population of cells in all plating densities was healthy looking with phase-dark, neurites and was non-pyknotic (from 40-65%, area G in Figure 2.7a-c). The highest proportion of this population was found at 3000 cells/mm^2 plating density. However, the population of cells with these morphological appearances neither absolutely overlapped with each other at any given plating density, nor followed the same trends as each other when the density was increased. Some phase-dark cells either showed pyknotic morphology, or produced no neurites, or both (Figure 2.7a-c, area D, E, and A). Some non-pyknotic cells were phase-bright, or had no neurites (Figure 2.7a-c, area F, E, and C) while cells with neurites were either phase-bright, or pyknotic (Figure 2.7a-c, area D, F, and B). Although sometimes the proportions of cells lacking one of these health-determining criteria were small, this changed in different plating densities. For example, there were a much higher percentages of non-pyknotic, phase-dark cells without neurites in low density culture (~12.82%, area E, Figure 2.7a) than in the medium and high density culture (< 3%, area E, Figure 2.7b, c).

As a result of the fact that populations of phase-dark cells, non-pyknotic cells, and cells having neurites did not always correspond with each other, determining cell viability in culture using one single criterion was difficult. On the other hand,

identifying viable cells with all these criteria would make my future experiments more tedious and might exclude some populations of cells that are in fact also healthy. To minimise the criteria used for determining viability and to choose a plausible criterion for measuring cell survival, I selected the nuclear morphological appearance as a criterion for future viability assessment. This was because 1) the study of nuclear change is a conventional method for assessing cell death (particularly apoptosis), as is well documented in many in types of investigations in tissue culture (Johnson, 1995) and 2) this criterion is consistent with results using nuclear fragmentation labeling (TUNEL) *in vivo*. In addition, unlike results from use of phase-brightness and production of neurites, the changes in proportions of pyknotic cells always correlated with changes of plating density. Also, as shown in Figure 2.7, the significant population of cells showing pyknotic nuclei were those with neurites and it is more likely that there were non-viable than vice-versa i.e. die after producing neurites.

However, as stated in section d), cells with intermediate chromatin condensation were sometimes difficult to classify into dead or live cells. I thus followed the change in percentages of these cells in high-plating density cells in 2h, 24h, and 48h cultures, to see whether the proportion of this intermediate group of thalamic cells either increased or decreased with time.

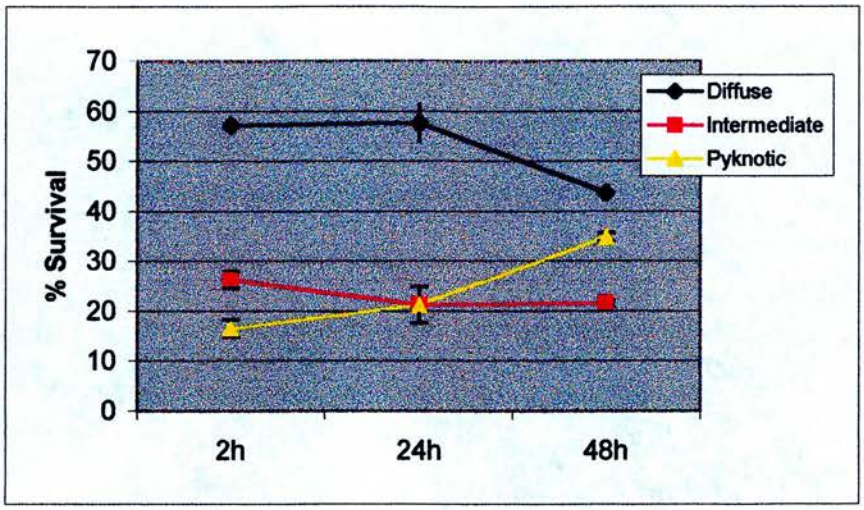
3.2) Monitoring thalamic cells in 2h, 24h, and 48h culture periods

In 2h high-density cultures, many cells had started to produce neurites (12.58 $\pm$ 1.52%, n = 5). Some also had growth cones. Most neurites were short and did not reach neighboring cells. By 24h, the majority of cells (95.54 $\pm$ 0.73%, n = 5) had produced neurites and formed contacts with other cells but, by 48hours, the percentage of cells which had produced neurites was marginally but significantly decreased (91.78 $\pm$ 2.37%, n = 5) ($p < 0.05$, reduced from the proportion of 24h cells which contained neurites).

The percentage of cells with pyknotic morphology increased over the period of culture, and the percentage of cells with diffuse chromatin was decreased after 2

Figure 2.8

Graph showing changes in the proportion of cells with different nuclear morphology after 2 hours, 24 hours, and 48 hours in high-density cultures (3000 cells/mm^2): Number of cells with pyknotic morphology is increased and number of cells with diffuse chromatin is decreased over time of culture. Percentages of cells with intermediate morphology show a small decrease.


70
60
50
40
30
20
10
0
% Survival
2h
24h
48h
Diffuse
Intermediate
Pyknotic

days in culture (Figure 2.8, see table below). The change in the percentage of intermediate cells showed the same trends as the change in the percentage of the population of cells with diffuse chromatin (it was reduced after 48h in cultures). The percentages of each cell population are shown in the following table:

Nuclear	% Survival, (n = 5 each)		
Appearance	2h	24h	48h
Diffuse	57.18 ± 0.93 %	57.67 ± 3.65 %	43.66 ± 1.28 %
Intermediate	26.31 ± 1.72 %	21.27 ± 3.66 %	21.54 ± 0.63 %
Pyknotic	16.51 ± 1.73 %	21.06 ± 0.45 %	34.79 ± 0.80 %

From the results of the observation of the population of intermediate cells at high plating density and following these cells for up to 2 days in culture, there is not enough evidence to classify these cells as non-viable. Moreover, when analysing cell cultures with the TUNEL reaction (see Figure 2.3e), all TUNEL positive cells were pyknotic cells (Figure 2.3e, in comparison with Heochst in Figure 2.3f). No cells with intermediate chromatin changes were labeled with TUNEL.

Discussion

1. Identification of thalamic structure using DNA fluorochrome

DNA binding fluorochromes stained only nuclei but not other structures like the cellular boundaries, cytoplasmic content, or neuronal fibers. This caused some problem in identification of many brain areas, which could normally be seen with Nissl staining (Schambra et al., 1992). However, in this study, I have confirmed that identification of dorsal thalamic nuclei using Hoechst is possible. With Hoechst, the nuclear structures can be differentiated by comparing the shape, boundaries and location of each thalamic nucleus with neighbouring structures. This method was used for further studies with *in vivo* fluorescent TUNEL.

2. TUNEL application in PCD studies

A conventional means of estimating PCD in developing brain is to compare number of live cells at two time-points and calculate cell loss (Hamburger, 1934; Hamburger and Levi-Montalcini, 1949). However, the major disadvantage of this method is that it does not always indicate that the cell loss is due to cell death, and it cannot tell whether the cell death was due to apoptosis or PCD (Oppenheim ,1999; Finlay and Pallas,1989). A more direct approach is to quantify the actual dying cells using techniques that detect properties of cells that are characteristic for apoptosis.

By labeling the ends of DNA strand breaks, the TUNEL method detects apoptotic cells that are undergoing DNA fragmentation. There are several advantages to methods that employ TUNEL (quoted from Coligan et al., 1995).

> "...1) Because the end labeling is performed inside the cells, TUNEL is by far the most direct method for analyzing cells in which DNA fragmentation is occurring.
>
> 2) Because DNA fragmentation is one of the earliest events in apoptosis, cells undergoing programmed cell death are detected at a very early stage in the process.

> 3) The accuracy of all-or-none labeling of TUNEL provides the clear distinction between cells undergoing apoptosis and the rest of the cells.
>
> 4) The TUNEL method is more sensitive than methods using vital dyes such as propidium iodide or Hoechst; hence DNA fragmentation may be detected 4-6hr before cell lysis/clearance (Kishimoto et al., 1995; Sellins and Cohen, 1987).
>
> 5) TUNEL staining is compatible with multiple-color staining, thus allowing definition of the cell surface phenotype of apoptotic cells (Kishimoto et al., 1995)
>
> 6) It is suitable most to tissue sections, thus allowing *in situ* viusaliasation of dying cells without having to use electron microscopic analysis. (Gavrieli et al., 1992)...."

TUNEL (Terminal-dideoxyribonucleotidyl Transferase (TdT) mediated dUTP nicked end labelling) was first introduced for detection of apoptosis by Gavrielli et al., (1992). However several researchers argued that TUNEL may not be specific or selective for apoptosis. Failure of TUNEL detection in the nervous system has also been reported (Herrup and Busser, 1995). Moreover, there were also reports that this method cannot distinguish between necrotic and apoptotic cells (Grasl-Kraupp et al., 1995; Charriaut-Marlangue and Ben-Ari, 1995). On the other hand, Didenko and Hornsby (1996) argued that TUNEL is specific enough for apoptosis since it selectively labels 3' overhanging DNA fragments which are characteristic of apoptosis. My studies revealed that all TUNEL positive cells were associated with pyknotic morphology and thus it can be assumed that TUNEL positive cells specifically label apoptotic cells, at least in the developing thalamus. Later findings (Chapter 3) also showed that these TUNEL positive cells were commonly found in embryonic and perinatal thalamic cells which suggested that PCD in the thalamus occurs via apoptosis.

One practical drawback of the TUNEL method is that it is more costly than other methods but it is still particularly useful for *in vivo* studies because it gives a high sensitivity and resolution. It can readily pick up dead cells which may be present in only a small quantity amoungst a high density of viable cells. In tissue

culture, however, this technique might not be necessary, as more dead cells are clearly visible due to the lack of cell clearance. Moreover, cells in culture are less packed and can be easily seen with normal DNA binding fluorochromes.

There are some advantages of using fluorescent detection for TUNEL over the conventional peroxidase colorimetric TUNEL detection in light microscopy. For example, fluorescent TUNEL can easily be counterstained with DNA binding fluorochromes so that 1) TUNEL positive cells can be quantified as a percentage of total cells in a particular region and 2) TUNEL positive cells can be identified as apoptotic cells from the pyknotic morphology visualised by DNA binding fluorochromes. Moreover, this produces higher resolution and is more accurate and reliable (Coligan, 1995). However, there is one drawback when fluorescent TUNEL is applied; the problem of autofluorescence. This artifact can be distinguished from TUNEL positive cells by using the following criteria: 1) autofluorescent cells exhibit a more yellowish-green color with a FITC filter and the signals can be seen with all fluorescence filters, while TUNEL positive cells appear as deep green color in FITC filter. 2) Most autofluorescent cells are non-neuronal derived (i.e. endothelial, enucleating blood cells, and pial cells) and the morphology of these cells is non-apoptotic (non-pyknotic).

As described in the Results section, some thalamic nuclei have no clear margin throughout the brain sections. It is difficult to quantify the total number of TUNEL positive cells in these thalamic nuclei individually. Thus, in subsequent experiments, I decided to determine the extent of cell death by quantifying TUNEL positive cells per total counted cells (with propidium iodide, PI, or blue DAPI counterstaining) in a window as a ratio of cell death per 1000 cells. This would allow me to investigate changes in the rates of cell death throughout the developmental period and to compare these with rates in other mutant tissues (see Chapter 3 and 6).

3. Setting up cell death criteria *in vitro*

3.1) Phase contrast microscopy and neurite production

As seen in the Results section, neither phase-dark cells nor cells with neurites were always healthy. Although many cells undergoing apoptosis were phase-bright, not all phase-bright cells were dying. Other reports indicated that several types of cells in cultures that are viable (such as embryonic motoneurons and olfactory receptor neurons) are phase-bright (Cohen and Wilkin, 1995). Davies and Wright (1995) also found that embryonic sensory neurons undergo morphological transition in culture. The neurons have small, spindle-shaped, phase-dark cell bodies and short neurites. Subsequently, they develop spherical, phase-bright cell bodies and extend long neurites.

With regard to the use of neurite production to determine viability, even though viability of thalamic cell culture is strongly correlated with the production of neurites (Lotto et al., 1997), some cells that are clearly pyknotic do not fully retract or disintegrate their neurites (see Results). Cell viability does not necessarily correspond with neurite growth. For example, in thalamic cells, factors influencing neurite outgrowth might be different from factors controlling cell survival. Previous studies have shown that, while diffusible molecules produced within cerebellum promote more thalamic cell survival than molecules within the cerebral cortex (Hisanaga and Sharp, 1990), the latter produce the reverse effect on neurite outgrowth (Lotto and Price, 1995). Thus, the measurement of neurites can not be used as a definite criterion for determining cell death *in vitro*.

3.2) Studies with DNA binding fluorochromes

The only advantage of combination staining over single nuclear staining is that it can distinguish necrotic cells (Coligan et al., 1995). Early- (blue) and late- (red) pyknotic cells are clearly dead and there is no need to differentiate the two. In embryonic thalamic cell culture, the percentage of necrotic cells is very low, and it is time-consuming to perform combination staining in the fresh specimen. In subsequent experiments, I thus ignored the necrotic population and used a single dye in fixed samples.

Nuclear analysis by Hoechst staining is well documented to be among the best, simplest, and most specific method for identification of apoptotic cells *in vitro* and flow cytometry (Ramachandra and Studzinski, 1995). Overall shrinkage and nuclear condensation can be recognised with a UV filter of a fluorescence microscope. Occasionally, these dead cells break down in to apoptotic bodies (fragments or vacuoles of chromatin). In my experiments, I have shown pyknotic cells stained by this means gave an indication of apoptosis as clear as with TUNEL labeling. The TUNEL method might be redundant for measuring apoptotic cells in cultures. Thus, I used this single staining method to determine cell viability in cultures. As for the classification of cells with intermediate chromatin condensation, I decided to classify these cells as viable cells as they did not show a clear indication of apoptosis and were not TUNEL positive.

4. Effects of plating density on thalamic survival

Thalamic cells require proper conditions to survive and grow in culture. In this experiment, high plating density provides a suitable environment to ensure the viability of E15 thalamic cells. The most probable explanation of this result is that increasing the plating density increases direct cell-cell interactions and/or the concentration of released neurotrophic molecules that are necessary for survival (Lotto et al., 1997). The increase in thalamic cell contact in high-density culture might increase neuronal activity (probably also synaptic transmission), and thus mediate neurotransmitter and/or neurotrophin release. This hypothesis is supported by two main previous reports: 1) Survival and neurite outgrowth of embryonic thalamus are promoted by potassium induced depolarisation in explant cultures (Magowan and Price, 1996). 2) Survival of thalamic cells at low density is equal to that of thalamic cells at high density if they were supplied by neurotrophins or FGFs (Lotto et al., 1997). However, we can interpret this result in another way, i.e. that thalamic neurons do not always require cell-cell contact as long as they are provided with enough trophic support. It is still unclear whether thalamic neurons at this age depend solely on their intrinsic trophic factors throughout development. Hisanaga and Sharp (1990) provided evidence that diffusible trophic factors from external

neuronal tissue can promote the survival of developing thalamic neurons. These questions will be further investigated in Chapters 3-5.

Chapter 3: Thalamic cell death studies and the role of the neurotrophins

Introduction

Programmed cell death (PCD) is a common phenomenon found in diverse structures of the developing nervous system such as sensory and autonomic ganglia, cranial and spinal motoneurons and cerebral cortex (Oppenheim, 1991). However, PCD in the thalamus is poorly understood (Spreafico et al., 1995). The time of PCD and the factors controlling PCD in the thalamus have to be identified. The first can be achieved by using TUNEL to analyse the quantity and distribution of PCD in the dorsal thalamus during development. However, the second is more complicated to investigate. The Neurotrophic Hypothesis proposes that neurotrophic factors, in particular neurotrophins, are factors that play a major role in regulating PCD. Prime candidates for controlling thalamic PCD are members of the neurotrophin family.

The members of the neurotrophin family include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Three high affinity tyrosine kinase (Trk) receptors have been isolated; NGF binds TrkA, BDNF and NT-4 bind TrkB and NT-3 binds Trks TrkA, B, and C (see also Chapter 1; Chao, 1992). These neurotrophins and their receptors are required for the survival and differentiation of specific neuronal populations during ontogeny, predominantly in the peripheral nervous system (Barbacid, 1995; Farinas et al., 1996).

In thalamus, all of these neurotrophins have been shown to promote survival of embryonic thalamic neurones at around E15 *in vitro* (Lotto et al., 1997). BDNF and NT-3 mRNAs are expressed from early midgestation. All of the Trk receptor mRNAs can also be first detected in the nervous system around early midgestation (Barbacid et al., 1991). In particular, *trkB* and *trkC* mRNAs, being widely expressed in many CNS regions and having significantly overlapping patterns, are thought to

play a regulatory role in CNS development (Ernfors et al., 1992; Merlio et al., 1992). In postnatal thalamus, strong expression of *trkB* and *trkC* (but not *trkA*) can be found in many defined nuclei (Ringstedt et al., 1993). The prenatal expression patterns of these mRNAs are not known. In this study, I carried out *in situ* hybridisation for mRNAs of TrkB and TrkC receptors (Barbacid, 1994, the probes detected both catalytic and non-catalytic forms of *trkB* and *trkC* mRNAs) in normal embryonic thalamus. There were two objectives for this: 1) to demonstrate the patterns of *trkB* and *trkC* expression in the embryonic thalamus (which has never been studied in detail) and 2) to study whether the hybridisation patterns correlate with the PCD patterns in the developing thalamus.

In vivo, it is not know whether neurotrophins regulate thalamic PCD. In postnatal life they control cell differentiation and plasticity (Spreafico et al., 1995; Alcantara et al., 1997; Maffei et al., 1992; Cabelli et al., 1995; Riddle et al., 1995). Despite early mRNA expression, the role of neurotrophins and their receptors particularly in PCD regulation in embryonic thalamus is still unclear.

Mutant mice lacking the genes encoding each of these neurotrophins or their receptors have been generated, to study the requirement for neurotrophin signaling in the survival of distinct PNS neuronal populations during embryonic development (Snider, 1994; Barbacid et al., 1995; Farinas et al., 1996). However, very few gross defects have been detected in the CNS of these mutant mice (Klein et al., 1993; Conover et al., 1995; Liu et al., 1995). Additionally, the effects of mutations in neurotrophin genes are generally subtler than mutations of their companion receptor genes. This suggests that some cells may obtain trophic support from more than one factor signaling through a common receptor pathway (Johnson and Oppenheim, 1994). The *in vivo* study of mutations of neurotrophin receptors often provides better evidence for their role.

In this chapter, I thus studied thalamic PCD in mice lacking TrkB and TrkC receptors at different ages throughout the embryonic and early postnatal periods. These periods are around the time when thalamic fibers innervate cortex (which

should be the period when thalamic cells develop a trophic requirement, according to the Neurotrophic Hypothesis). I analysed PCD in the thalamus of mice heterozygous for targeted mutations in trkB or in trkC sequences encoding the catalytic domains of the TrkB or TrkC tyrosine kinase receptor isoforms (Klein et al., 1993; Klein et al., 1994). Mice lacking TrkB were first generated by targeted mutation. A phosphoglycerate kinase 1 (PGK1)-neo cassette (McBurney et al., 1991) were inserted to disrupt the domains III-V (exon K2, Klein et al., 1993) of the tyrosine kinase domain. Cells were dual selected in the presence of G418 and gancyclovir and screened by the PCR method. The selected cloned ES cells were then used to produce heterozygous *trkB*+/- mice (Klein et al., 1993). A similar method was done for targetting *trkC*, the inserted PGK1-*neo* cassette disrupts K2 exon and is spliced out and leading to an aberrant transcript that contains a frame shift mutation between exons K1 and K3. This *trkC* mutation results in the synthesis of abnormal TrkC receptors of 667 amino acids (against original 825 residues)(Klein et al., 1994).

Later in this chapter, to strengthen the evidence, several sets of cell culture experiments are described which show that at least one neurotrophin (BDNF) and its receptor (TrkB) are likely to be involved in thalamic PCD at a particular period of development. These experiments were done in collaboration with a postdoctoral fellow, R. B. Lotto. First, we tested whether thalamic cells in culture have a better survival when provided with certain neurotrophins and/or trophic factors from medium preconditioned with cerebral cortex. Then we further tested the neurotrophic requirement of thalamic cells by applying the neurotrophic signaling kinase inhibitor, K252a (Tapley et al., 1992; Kang and Schuman, 1995), and specific blockers to TrkA, TrkB and BDNF.

Materials and methods

Animals/tissue preparation

1) Mice prepared for in situ hybridisation

Four E19 C3H pregnant mice were used in this study. E19 embryonic brains were dissected and processed histologically as described in Chapter 2. Coronal sections were cut at 7μm and mounted on 0.1% TESPA (in acetone solution) coated slides (instead of using normal poly-$_L$-lysine). All procedures were performed under RNase-free conditions, i.e. gloves were worn to avoid enzyme contamination from the investigators to the tissue.

2) Transgenic mice prepared for TUNEL studies

Heterozygous mice for a null mutation of trkB or trkC were mated to give wild type, heterozygous, and homozygous offsprings. Embryos were removed at the assigned stage of pregnancy. Parts of the embryos (either legs or tails) were removed for PCR genotyping. Brains were obtained from E13, E15, E17, E19, P1, and P4 mice. Embedding was done as described in Chapter 2. Coronal sections were cut at 7μm and mounted on poly-$_L$-lysine coated slides.

Riboprobe preparation

Plasmids containing sequences recognising *trkB* and *trkC* extracellular domains were provided by Julia Clausen & Tom Pratt (obtained from Bristol-Myers Squibb).

The *trkB* antisense riboprobe was transcribed from a 0.5kb fragment derived from the linearised pFRK-16 plasmid (Klein et al., 1989) using EcoR V restriction enzyme and T7 RNA polymerase. Sense probes were generated from the same plasmid for controls, using Sma I restriction enzyme and SP6 RNA polymerase.

The *trkC* antisense riboprobe was transcribed from a 0.5kb fragment derived from the linearised pFL-25 plasmid (Klein et al., 1991) using EcoR I restriction enzyme and SP6 RNA polymerase. Sense probes were generated from the same plasmid for controls, using Hind I restriction enzyme and T7 RNA polymerase.

Both *trkB* and *trkC* probes would detect mRNAs encoding both truncated and full length receptors. In each case, 1μg of each of these plasmids was used as a DNA template and linearised with appropriate restriction enzymes according to methods provided by the manufacturing company (Roche). The DNA was extracted by standard phenol-chloroform extraction and ethanol precipitation.

The *in vitro* transcription was carried out by mixing the extracted template with appropriate RNA polymerase (see above) 2U/μl in 20mM Tris-HCl, pH8.0, 6mM $MgCl_2$, 10mM DDT, 10mM NaCl, 2mM Spermidine, RNase Inhibitor 1U/μl, 1mM each ATP, CTP and GTP, 0.65mM UTP and 0.35mM DIG-11UTP at 37°C for 2h. The reaction was completed by an incubation with 2U/μl of RNase-free DNase (Roche) at 37°C for 15 minutes and ethanol precipitation in 20mM EDTA pH8.0, 0.1 volume LiCl at –70°C for 1h. Following centrifugation for 5 minutes at 4°C, samples were washed in 70% ethanol, dried, resuspended in 100μl DEPC-treated water and stored at –70°C. Labeled RNA was quantified by dot-blot analysis (Roche). Only transcription reactions yielding 1μg/ml RNA were used as riboprobes for *in situ* hybridisation.

In situ hybridisation

For prehybridisation steps, brain sections on slides were dewaxed, rehydrated, and washed in SSPE solution. They were then treated with 20μg/ml proteinase K, post-fixed with chilled 4% paraformaldehyde, 0.2M HCl, and acetylated (to reduce background) with 0.5% acetic-anhydride in 0.1M triethanolamine (TEA). At the hybridisation steps, I added 1-2μl DIG-riboprobes (100-200ng) in 60μl hybridisation mix (50% formamide, 10% dextran sulfate, 0.5%

100x Denhardts, 0.2mg/ml tRNA, and 0.05% SDS in SSPE) on each slide. The reactions were incubated overnight at 50°C in a humidified atmosphere. On the following day, slides were washed with 2x SSC (10 minutes, 50°C), 2x SSC in 50% formamide (45 minutes, 65°C), 4x SSPE (5 minutes, 50°C), 20ug/ml RNAseA in 4x SSPE (30 minutes, 37°C), 2x SSC in 50% formamide (45 minutes, 65°C), and then in pre-warmed 50°C 2x SSC. The hybridised slides were processed through DIG antibody detection steps. Tissues were washed with 0.1% Tween 20 in PBS, 1% blocking buffer, and incubated in 1:5000 anti-DIG-AP (alkaline phosphatase) antibody solution overnight. 2% NBT/BCIP stock solution in alkaline phosphatase buffer was applied for color detection. Tissues were then counterstained by 1% PyroninB and mounted. Expression was analysed under the microscope. Anatomical descriptions in this study were according to Paxinos et al. (1994) and Schambra et al. (1992).

Mutant Genotyping

Frozen foetal/pups' lower trunks (E13), legs (E15-E17), and tails (E19-P4) were digested by protinase K, followed by standard phenol-chloroform-isoamyl DNA extraction, PCR, and gel eletrophoresis (2% agarose) according to Schimmang et al. (1995). Primers used are summarised below.

PCR Products:

Knockout	Allele	Primers 5'/3' (Oswel seq name)*	PCR product size (bp) (approx.)
TrkB	Normal allele	TRK1 5' common(T6966)/TRK 3' WT(T6967)	900
	Targeted allele	TRK1 5' common(T6966)/TRK 3' Targetted(T6968)	1000
TrkC	Normal allele	TRK4 5' common(T6969)/TRK 3' WT(T6964)	400
	Targeted allele	TRK4 5' common(T6969)/TRK 3' Targetted(T6965)	500

TrkB: a) Common 5' primer = 5'- TCGCGTAAAGACGGAACATGATCC-3'
b) 3' WT primer = 5'- AGACCATGATGAGTGGGTCGCC -3'
c) 3' Targetted primer = 5'- GATGTGGAATGTGTGCGAGGCC -3'

TrkC: a) Common 5' primer = 5'- CTGAAGTCACTGGCTGAGTCTGGG-3'
b) 3' WT primer = 5'- GTCCCATCTTGCTTACCCTGGAGG -3'
c) 3' Targetted primer = 5'- CCAGCCTCTGAGCCCAGAAAGC -3'

TUNEL technique & identification of thalamic nuclei

TUNEL protocol was performed on sections as described in Chapter 2. DNase-induced (10μg/ml) positive control slides and negative control (TdT omitted) slides were run together with other slides in every single experiment.

TUNEL cell counts

Counts were made in the most distinctive area of each thalamic nucleus of each slide. At 40x magnification, TUNEL positive cells were identified by green fluorescence under the fluorescein filter (they differ from yellowish green cells, which are autofluorescent background, see Chapter 2 - Figure 2.2) and counted within the grid window (10 x 10 square area). Apoptosis was further confirmed by nuclear morphology (pyknosis and/or apoptotic bodies) under DAPI or PI staining. The numbers of TUNEL positive nuclei and of total nuclei (obtained from counting DAPI or PI stained nuclei) per grid window were counted. The ratio of the number of

TUNEL positive cells to the total number of cells in a thalamic nucleus was expressed per 1,000.

There are four major methods of cell counting in histologically sectioned material: 1) serial reconstructions; 2) stereological methods; 3) assumption-based methods; and 4) profile counts (for detail see Coggeshall and Lekan, 1996). The method of cell quantification used in this study was regarded as 'densities and ratios' type of profile counting method. This method is one of the simplest and the mostly commonly used methods. However it can be biased and the accuracy depends on whether situations that cell size, shape and the orientation of the compared profiles are not varied. The two populations of cells (TUNEL positive and non-labeled cells) counted in this study were similar in size (3-6μm, and 7-10μm) so this method was used in this study. Statistical analysis was carried out by Students' t-test.

Numbers of embryos used in the TUNEL experiment are summarised below.

Ages	E13	E15	E17	E19	P1	P4
Wild type mice	3	4	5	5	3	3
TrkB-/- mice	3	4	3	4	3	4
TrkC-/- mice	3	3	3	3	3	3

Tissue culture

Detailed methods are described in Chapter 2. In this Chapter, cells were plated at high density (4,500 cells/mm^2), except in experiments testing the blocking effect of BDNF antibody where some were plated also at low density (1,000 cells/mm^2) (Figure 3.6). In each culture well, numbers of live and dead cells in 10 randomly selected 0.2 x 0.2mm windows were counted blind to give the percentage

of viable neurons; data from multiple wells under each condition were combined to give averages.

Addition of substances to the cultures

This work was done in collaboration with R.B. Lotto. The following procedures were mostly done by R.B. Lotto. The experiment with neutralising the effect of BDNF by adding anti-BDNF antibody was done by me. CCM was obtained by dissecting the E19 cortex, sectioning at 500μm x 500μm using a McIlwain tissue chopper, culturing the slices on Costar Transwell inserts (40 slices in 500μl of medium) for 24h at 37°C, and removing the medium, which was stored at –20°C. Control medium was also incubated for 24h and stored at –20°C. After thalamic cells had been cultured for 3 days, 60μl of medium was removed and replaced with 70μl of either control medium or CCM and 10μl of phosphated buffered saline containing K252a, one of the Trk IgGs, anti-BDNF, turkey serum, one the neurtrophins or nothing (final concentration are given with results). Extra volume was replaced to compensate for slight evaporation.

Results

I. In vivo

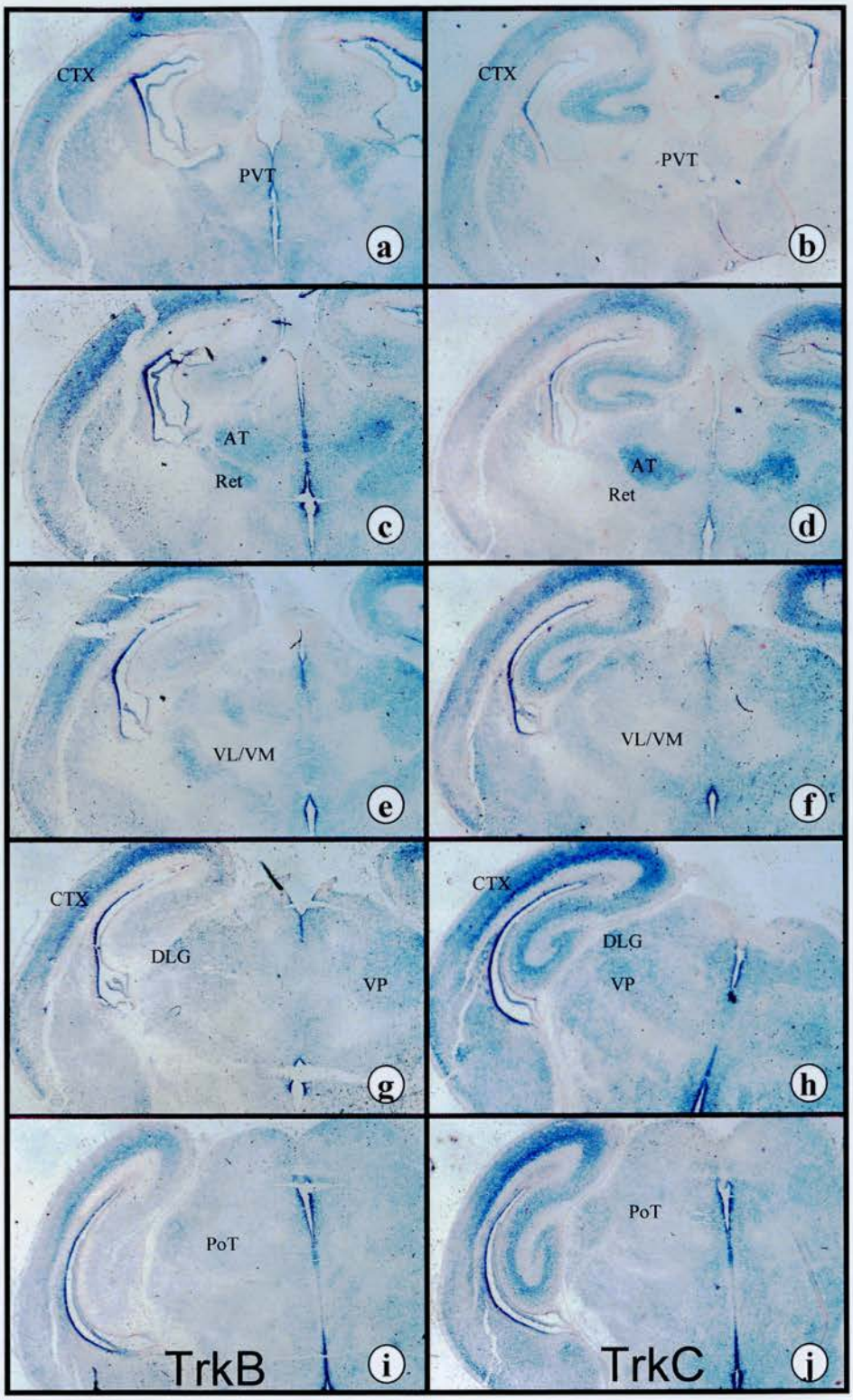
trkB and *trkC* mRNAs are expressed in several distinct embryonic thalamic nuclei in restricted patterns

The trkB expression pattern in the embryonic brain at E19 was heterogeneous (Figure. 3.1, left column). Strong expression was found throughout the cerebral cortex and in certain thalamic nuclei. In thalamus, most thalamic nuclei were labeled with the *trkB* probe. The highest expression was found in the anterior (including anteroventral/anteromedial) thalamic nuclei (AT), the reticular nucleus (part of the ventral thalamus), and the dorsal lateral geniculate nucleus (DLG). Slightly less but still strong expression of trkB was found in the ventral posterior thalamic nuclei (VP, ventroposteromedial and ventroposterolateral nuclei – VPM/VPL) and the paraventricular thalamic nuclei (PVT). The rest of the thalamic nuclei express *trkB* at a low level, except the ventral thalamic nuclei (VT, ventrolateral and ventromedial – VL/VM) which are both devoid of *trkB* expression.

The *trkC* mRNA probe strongly labeled the cerebral cortex and the hippocampus (Figure. 3.1, right column). Posterior cortex seemed to express *trkC* more strongly than the anterior area. In the thalamus, the distribution of *trkC* expression was similar to that of *trkB* but was different in intensity. The highest *trkC* expression was found in the anterior thalamic nuclei (AT). Ventral posterior nuclei (VP) were also labeled relatively strongly. Similar to that of *trkB*, *trkC* expression was not detected in the ventrolateral or the ventromedial nuclei. In contrast, *trkC* was not expressed in the reticular nucleus. The labeling intensity in the dorsal lateral geniculate nucleus was high but slightly less than that of *trkB*. The expression in the paraventricular nucleus was low. However, posterior and lateral dorsal nuclei expressed *trkC* at higher levels than *trkB*.

Figure 3.1

In situ hybridisation photomicrographs of *trkB* and *trkC* mRNA in E19 thalamus and cortex. In (a) an anterior coronal sections cuts across paraventricular thalamic nucleus, and *trkB* mRNA is expressed in paraventricular thalamic nucleus (PVT) and cerebral cortex. (b) Adjacent section shows *trkC* expression patterns. In (c) and (d) sections cuts across anterior group of thalamic nuclei. Strong *trkB* expression is found in anterior group of thalamic nuclei (AT). *trkB* (c), but not *trkC* (d), is also strongly expressed in the reticular thalamic nuclei (Ret) of the ventral thalamus. (e) and (f) are further sections across anterior and ventral groups of thalamic nuclei. VL/VM (ventrolateral and ventromedial nucleus) is void of *trkB* (e) or *trkC* (f) expression. In (g) and (h), more posterior sections cut across VL/VM, ventral posterior (VP), and dorsal lateral geniculate (DLG) nucleus of the dorsal thalamus. Strong expressions of both trkB (g) and trkC (h) mRNAs are in DLG and VP. In sections with the most posterior part of the thalamus, moderate expressions of both *trkB* (i) and *trkC* (j) mRNAs are found in posterior thalamic nucleus (POT). *trkC* mRNA is also strongly expressed in hippocampus and the cortex (b, d, f, h and j).


CTX
PVT
a
CTX
PVT
b
AT
Ret
c
AT
Ret
d
VL/VM
e
VL/VM
f
CTX
DLG
VP
g
CTX
DLG
VP
h
PoT
TrkB
i
PoT
TrkC
j

500μm

If the functions of the proteins depend on the level of the mRNA expression, the strong and overlapping expression of *trkB* and *trkC* would indicate that these two receptors may play an important role in the development of the anterior thalamic nuclei. While *trkB* is likely to regulate development of the dorsal lateral geniculate nucleus and the ventroposterior complex, *trkC* is likely to function in the posterior nucleus. These functions may also include PCD regulation.

Cell death in thalamus occurs with a peak soon after birth

Apoptosis in the normal thalamus was first detected at the earliest of the embryonic ages studied, i.e. E13. At this age, although the thalamus has developed and is identifiable, subdivision into the individual thalamic nuclei is not possible. Ratios of dead to live cells were thus measured in the whole dorsal thalamus (DT). The cell death rate was very low at E13: 1.95 ($\pm$ 0.26 s.e.m.) cells per 1000 cells (Figure 3.2a). From E15, some thalamic nuclei were distinguishable (at this age, I differentiated the thalamic nuclei into paraventricular nucleus, ventral nuclear group, dorsal lateral group, and posterior nuclear group). The amount of cell death remained low in almost every nucleus aged E15, E17 and E19, with the apoptotic ratio being 0.5-2 cells per 1000 cells. Among these thalamic nuclei, ventral medial/ventral lateral groups had the lowest rates of cell death at all ages ($\sim$ 0.3 apoptotic cells/1000cells) (Figure 3.2b-d, 3.5c). Slight increases in cell death were found in the posterior thalamic nucleus and the dorsal lateral geniculate nucleus at E19 (3.6 and 2.7 cells/1000cells, respectively).

Soon after birth, there was a general marked reduction in cell density, and cell death was more clearly observed (Figure 3.2-3.4). At P1, increased cell death was found in all thalamic nuclei, particularly in the ventroposterior nuclei (Figure 3.3a, 3.5e&g). I found as many as 55 apoptotic cells per 1000 cells in this VP region, the highest cell death ratio observed in this study. The average cell death rate in the whole thalamus (Figure 3.4b) at this age was 13.11 cells per 1000 cells ($\pm$ 1.51 s.e.m.). The second highest cell death rate was found in posterior nucleus (16 dead cells per 1000 cells). Again the lowest cell death rate was observed in the ventral nuclei (around 4/1000 cells).

At P4, the last age studied, cell death was still high. 10.6 apoptotic cells per 1000 cells was the average. The ventral posterior nuclear group (VPL/VPM) had no longer maintained its high cell death rate (reduced to 14.76 $\pm$ 6.26 cells/1000cells). A marked increase in cell death was found in the dorsal lateral geniculate (DLG), with a rate of 26 cells per 1000 cells (comparing to just about 9 cells per 1000 cells at E19) (Figure 3.3b)

A summary of the developmental PCD in thalamus as a whole is shown in Figure 3.4b. The rate of PCD in thalamus as a whole increased gradually as the thalamus matured and reached the peak at P1 as a result of the increase in PCD in every thalamic nucleus at this age, although rates of cell death among thalamic nuclei are different.

Roles of TrkB and TrkC in thalamic PCD

The gross morphology of the *trkB-/-* and the *trkC-/-* brain was relatively normal at all ages studied. In postnatal animals after day 4, trkB and trkC homozygous mutant mice showed reduced body size. *trkC-/-* neonates also showed some defects in movement possibly due to abnormal muscle coordination. The patterns of cell death in the *trkB* and the *trkC* thalamic mutants were generally similar to wild types but there were noticable exceptions. In *trkC* knockouts, unexpectedly, I found a slightly lower cell death rate at E13. Cell death in E13 trkC knockout thalamus was only 0.3 ($\pm$ 0.2 s.e.m) cells per 1000 cells which is significantly lower than wild type thalamus ($p < 0.05$, Student's t-test) (Figure 3.2a, 3.6a-c). On the other hand, there was no significant difference in apoptosis of *trkC-/-* thalamus compared to that in the thalamus of wild types in all other areas at the rest of the ages studied (E15-P4) (Figure 3.2, 3.3 and 3.4).

In *trkB* knockouts, cell death started to rise at E17. The most dramatic increase in apoptosis was found in the DLG (Figure 3.2, 3.4a, 3.6d-e) (13 $\pm$ 0.7/1000 cells compared to 1.5 $\pm$ 0.9/1000 cells in E17 wild type, $p < 0.01$). This change in the rate of cell death in the DLG alone, however, was significant enough to increase the

Figure 3.2

Graphs showing number of TUNEL positive cells per 1000 cells against different areas of wild type, *trkB-/-* and *trkC-/-* thalamus at different embryonic ages.

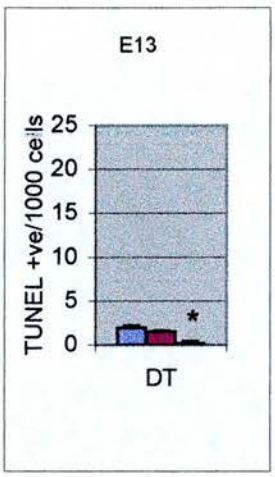
(a) Thalamic cell death at E13: the ratios of TUNEL positive cells are only counted in the whole dorsal thalamus as the nuclei have not differentiated. A small reduction in cell death is observed in TrkC knockout thalamus ($p < 0.05$).

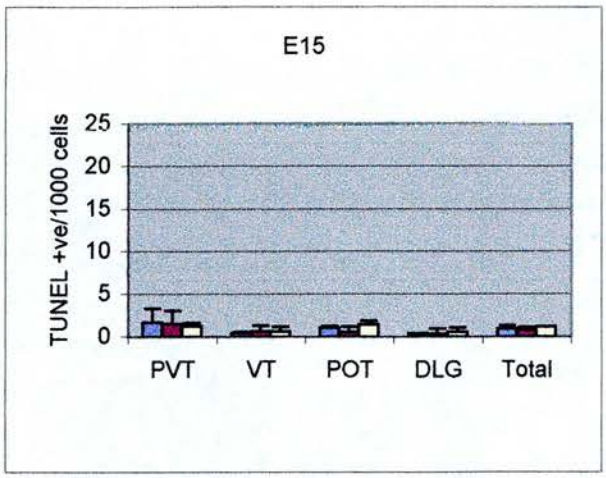
(b) Thalamic cell death at E15: some thalamic nuclei have differentiated. Cell death at this age is low and no significant difference in the mutant cell death is found at this age.

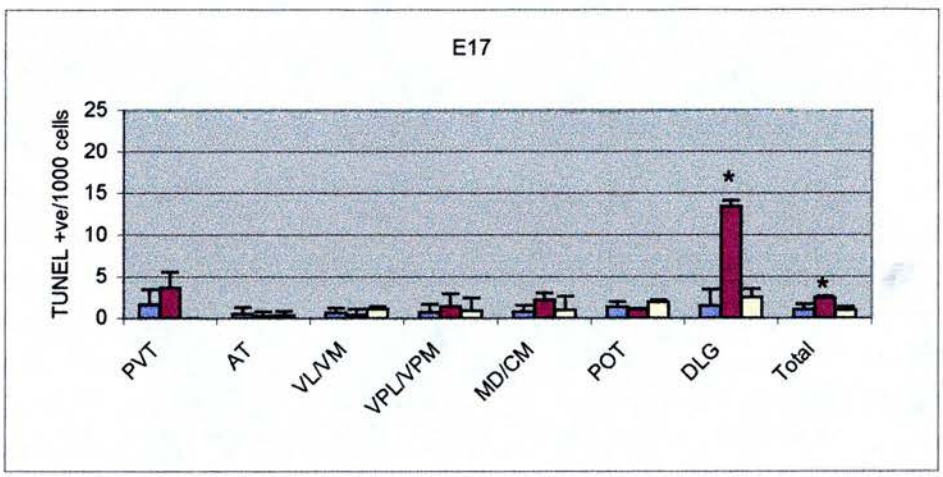
(c) Thalamic cell death at E17: average cell death in each nucleus is below 5 cells/1000 cells. A significant increase in cell death is observed in *trkB* mutant dlg ($p < 0.05$) which also makes the average cell death in this mutant thalamus increase as a whole ($p < 0.05$).

(d) Thalamic cell death at E19: average wild type cell death in each nucleus remains low. A small but significant increase in cell death is observed in AT, VPL/VPM, and in the DLG of the *trkB* mutant (all $p < 0.05$) but these do not make the average cell death in this mutant thalamus increase as a whole.

Abbreviations: WT, Wild type mice; trkB (-/-), tyrosine kinase B receptor knockout mice; trkC (-/-), tyrosine kinase C receptor knockout mice, DT, Dorsal thalamus; PVT, Paraventricular thalamic nucleus; VT, Ventral thalamic nuclei (including VL/VM – Ventrolateral/Ventromedial and VPL/VPM – Ventroposterior lateral/Ventroposterior medial nuclei); AT: Anterior thalamic nuclei; MD/CM, Mediodorsal and centromedial group of thalamic nuclei; POT, posterior thalamic nuclei; DLG, Dorsal lateral geniculate nucleus; Total = average rate of cell death in the whole thalamic area.


E13
TUNEL +ve/1000 cells
25
20
15
10
5
0
*
DT


E15
TUNEL +ve/1000 cells
25
20
15
10
5
0
PVT
VT
POT
DLG
Total


E17
TUNEL +ve/1000 cells
25
20
15
10
5
0
*
*
PVT
AT
VL/VM
VPL/VPM
MD/CM
POT
DLG
Total

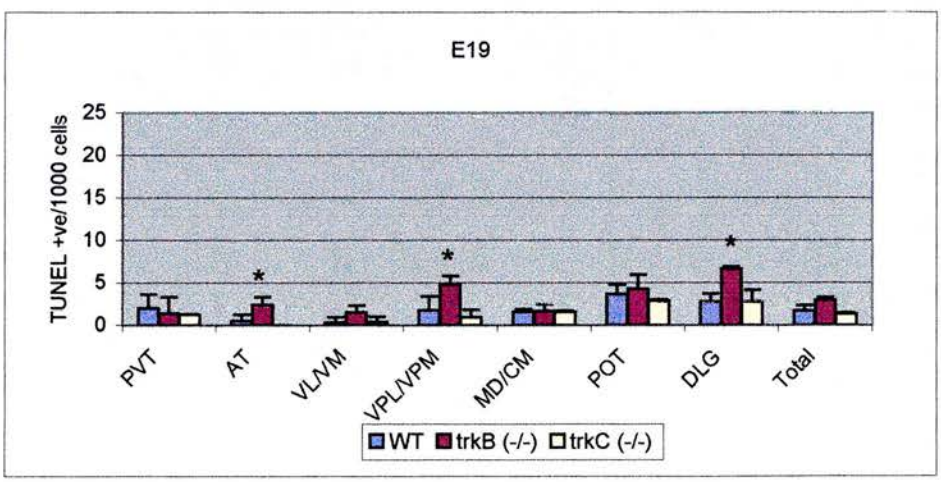

E19
TUNEL +ve/1000 cells
25
20
15
10
5
0
*
*
*
PVT
AT
VL/VM
VPL/VPM
MD/CM
POT
DLG
Total
WT
trkB (-/-)
trkC (-/-)

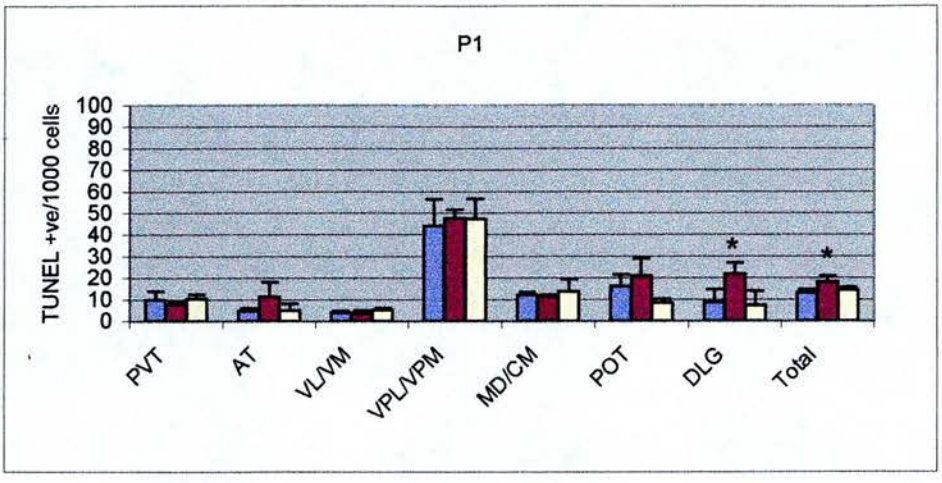
Figure 3.3

Graphs showing number of TUNEL positive cells per 1000 cells in different areas of the thalamus at postnatal days 1 and 4.

(a) Thalamic cell death at P1: The rate of cell death goes up to at least 10 cells/1000 cells. High rates of cell death in VPL/VPM are found in all wild type, *trkB-/-*, and *trkC-/-* animals. Significant increase in cell death is still found in trkB dlg comparing with wild type.

(b) Thalamic cell death at P4: The rate of thalamic cell death remains high but there is a reduction in cell death in VPL/VPM. No significant difference is found in the mutants.

Abbreviations: see Figure 3.2.


P1
TUNEL +ve/1000 cells
100
90
80
70
60
50
40
30
20
10
0
PVT
AT
VL/VM
VPL/VPM
MD/CM
POT
DLG
Total
*
*

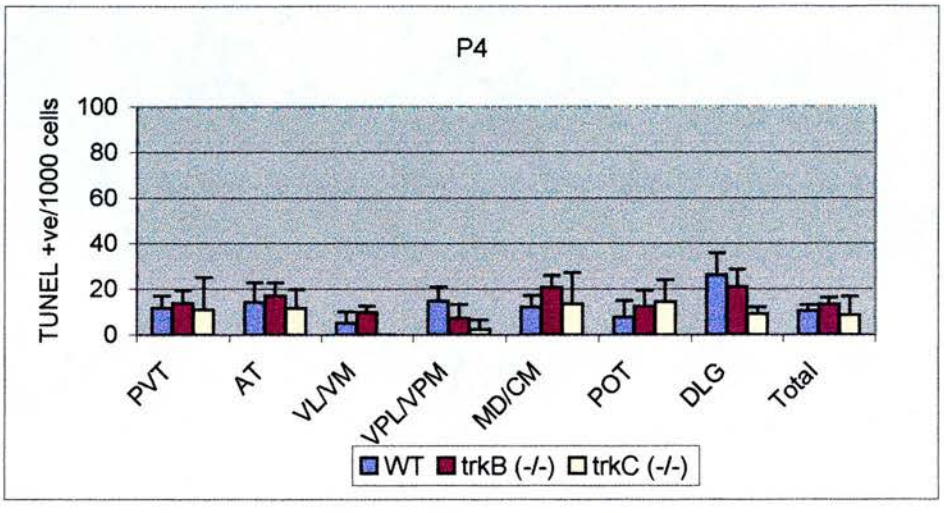
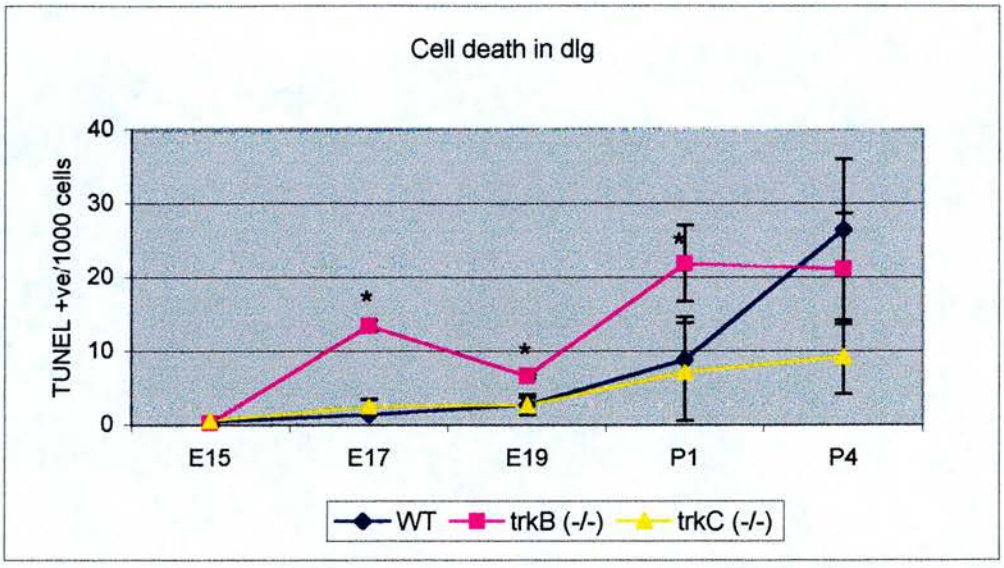

P4
TUNEL +ve/1000 cells
100
80
60
40
20
0
PVT
AT
VL/VM
VPL/VPM
MD/CM
POT
DLG
Total
WT
trkB (-/-)
trkC (-/-)

Figure 3.4

Summary graphs showing TUNEL positive cells per 1000 cells in wild type and mutant thalamus plotted against developmental ages.

(a) Cell death in dorsal geniculate nucleus (dlg): increased cell death is found in *trkB-/-* dlg (red) from E17 to P1. At P4, a rise in wild type cell death goes up to equal the level of PCD in the mutant (black line).

(b) Average cell death as in a whole thalamus of the wild type and mutants: all increase at P1.


Cell death in dlg
TUNEL +ve/1000 cells
40
30
20
10
0
E15
E17
E19
P1
P4
WT
trkB (-/-)
trkC (-/-)

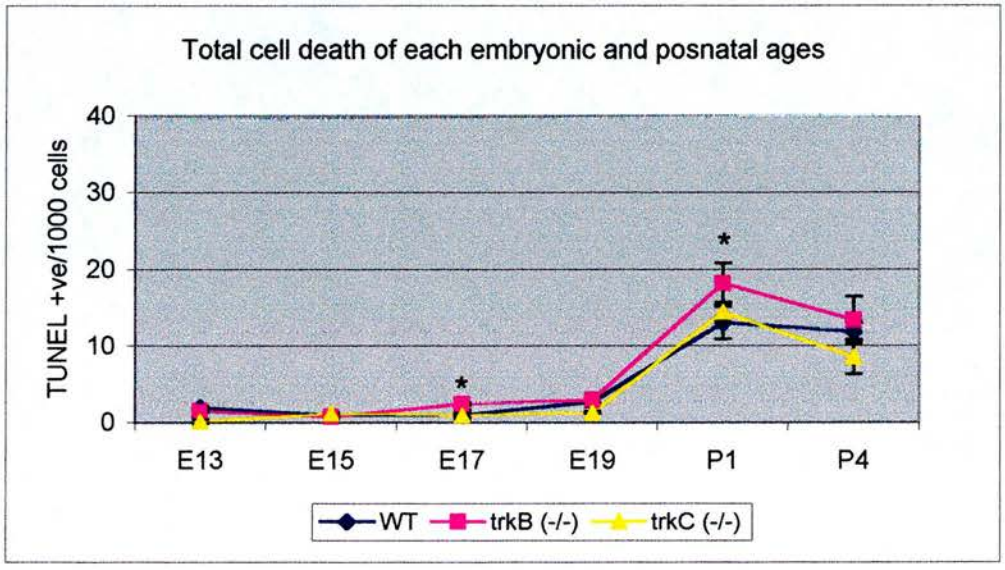

Total cell death of each embryonic and posnatal ages
TUNEL +ve/1000 cells
40
30
20
10
0
E13
E15
E17
E19
P1
P4
WT
trkB (-/-)
trkC (-/-)

Figure 3.5

Photomicrographs showing distributions of TUNEL positive cells in wild type P1 thalamus: TUNEL in (a) paraventricular thalamic nucleus (PVT), (b) Anterior thalamic nuclei (AT), (c) ventrolateral/ventromedial thalamic nuclei (VL/VM), (d) mediodorsal and centromedial group of thalamic nuclei (MD/CM), (e) ventroposterolateral/ventroposterior medical thalamic nuclei (VPL/VPM), (f) posterior thalamic nuclei (POT). Dorsal lateral geniculate nucles is not shown here. At higher magnification, more apoptotic cells is found in ventral posterior nuclei (g). Red arrows depict TUNEL positive cells.

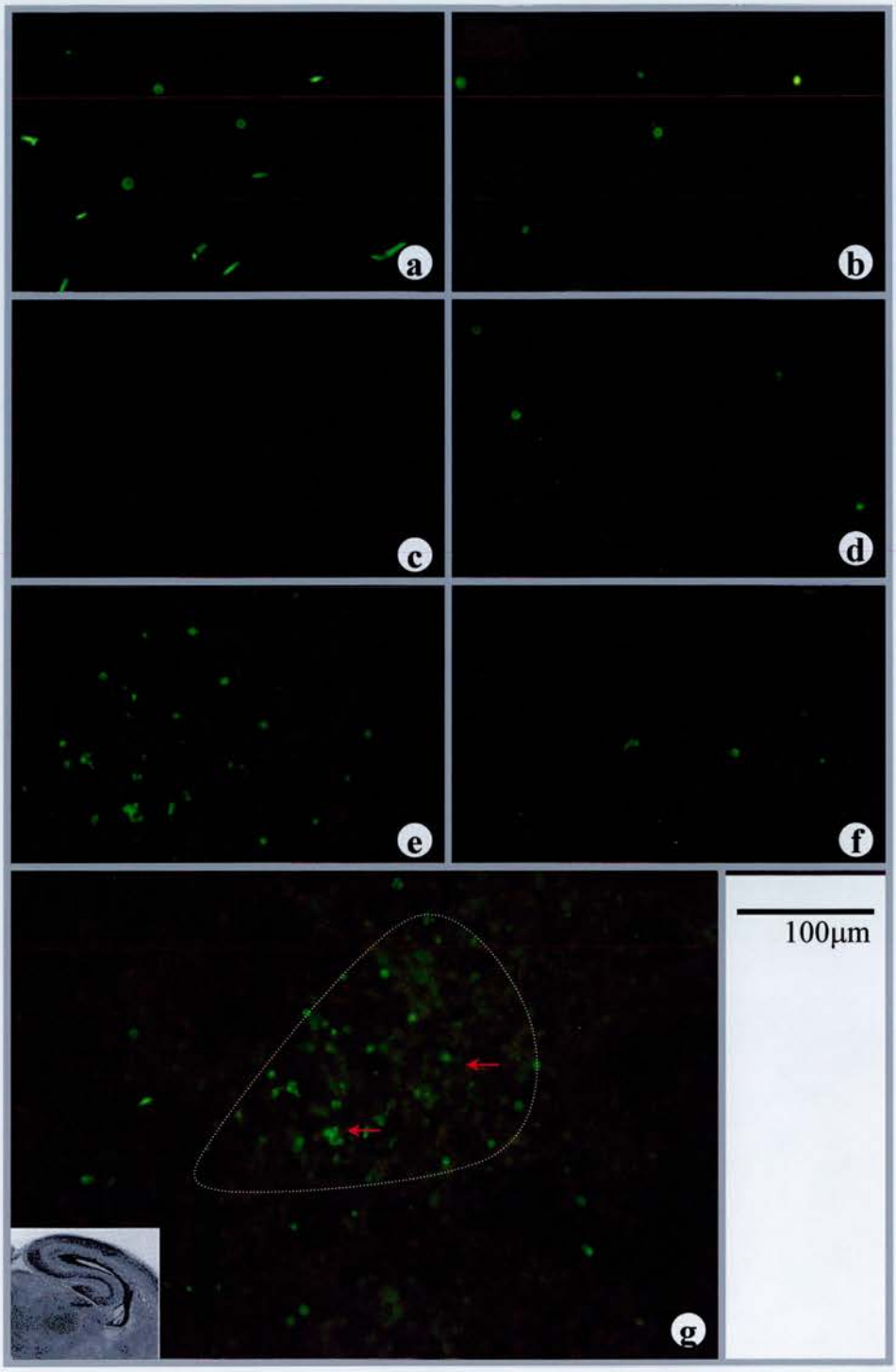
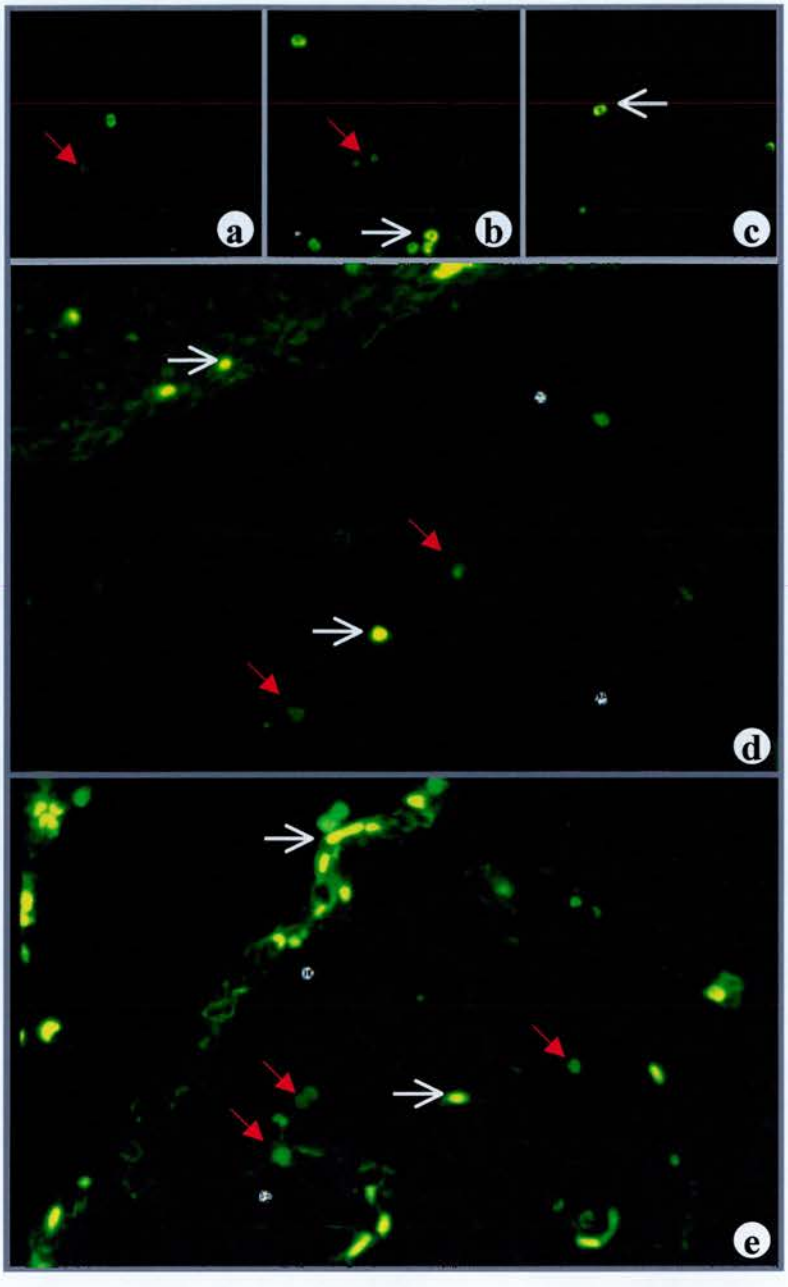

a
b
c
d
e
f
g
100μm

Figure 3.6

Photomicrographs showing distributions of TUNEL positive cells in E13 and E17 thalamus of the wild types and the mutants.

(a)-(c) E13 thalami (wild type, *trkB-/-*, and *trkC-/-* accordingly): a small reduction in the rate of cell death is observed in trkC knockout thalamus (c).

(d) and (e) E17 dorsal geniculate nucleus: in trkB mutants (e), there are more TUNEL positive cells than wild type (d). Red arrows depict TUNEL positive cells while white arrows are autofluorescent artifacts.



100μm (a-c)

50μm (d, e)

rate of E17 cell death in thalamus as a whole in the mutant. If I omitted the result in DLG, there was no significant increase in the rate of cell death in the mutant. At E19, the rate of cell death in *trkB-/-* DLG declined but remained significantly higher than in the wild type DLG (6.6/1000 cells, compared to 2.7/1000 cells in wild type, $p < 0.05$). Other regions were also found to contain more apoptosis in *trkB-/-* thalamus, i.e. anterior thalamic nuclei (2.4/1000 cells vs. 0.5/1000 cells in wild types, $p < 0.05$), and ventral posterior nuclei (4.79/1000 cells vs. 1.75/1000 cells in wild types, $p < 0.001$). The total amount of cell death in *trkB* mutant thalamus was therefore increased ($p < 0.05$; Figure 3.4a). In contrast to the previous report (Alcantara et al., 1997), the increased cell death in ventral posterior nuclei in the knockouts was found only at E19, and the high rate was not extended to later postnatal ages.

At postnatal ages, there was still an increase in cell death of *trkB* knockout DLG (21.8/1000 cells vs. 8.78/1000 cells in wild types, $p < 0.05$), but overall cell death at P1 was not significantly increased. At P4, there was no significant difference in cell death in any nucleus.

II. *In vitro*

Thalamic neurons at the age of cortical innervation *in vitro* require neurotrophic support

I have previously shown that E15 thalamic viability in 24h culture in enhanced if cells are plated at high-density. However, viability of E19 thalamic cells was poor under the same condition (high-density) (Figure 3.7a). When E15 thalamic cells were cultured at high-density up until an age equivalent to E19 or the time of birth (4-6 DIV), most cells died (these cells normally survive only up to 3 DIV). This suggested that intrinsic factors presented in the high-density environment were no longer sufficient for the late thalamic neurons. Thalamic cells at this stage are known to depend on target(cortical)-derived trophic support. We examined this trophic influence by adding medium preconditioned with cortical explants (CCM) and found that the CCM maintained E15 thalamic cells in culture for up to 6 DIV (Figure 3.7a). Detail investigation of basic properties and specificity of the conditioned medium

Figure 3.7

Graphs showing thalamic viability in high plating density of 5-day culture with treatments of cortical conditioned medium, neurotrophins and their blockers (taken from R.B. Lotto).

(a) E15 and E19 thalamic viability without treatment: E15 thalamic cells survive up to 3 days and die soon after. One-day culture shows a poor viability of E19 thalamic cells.

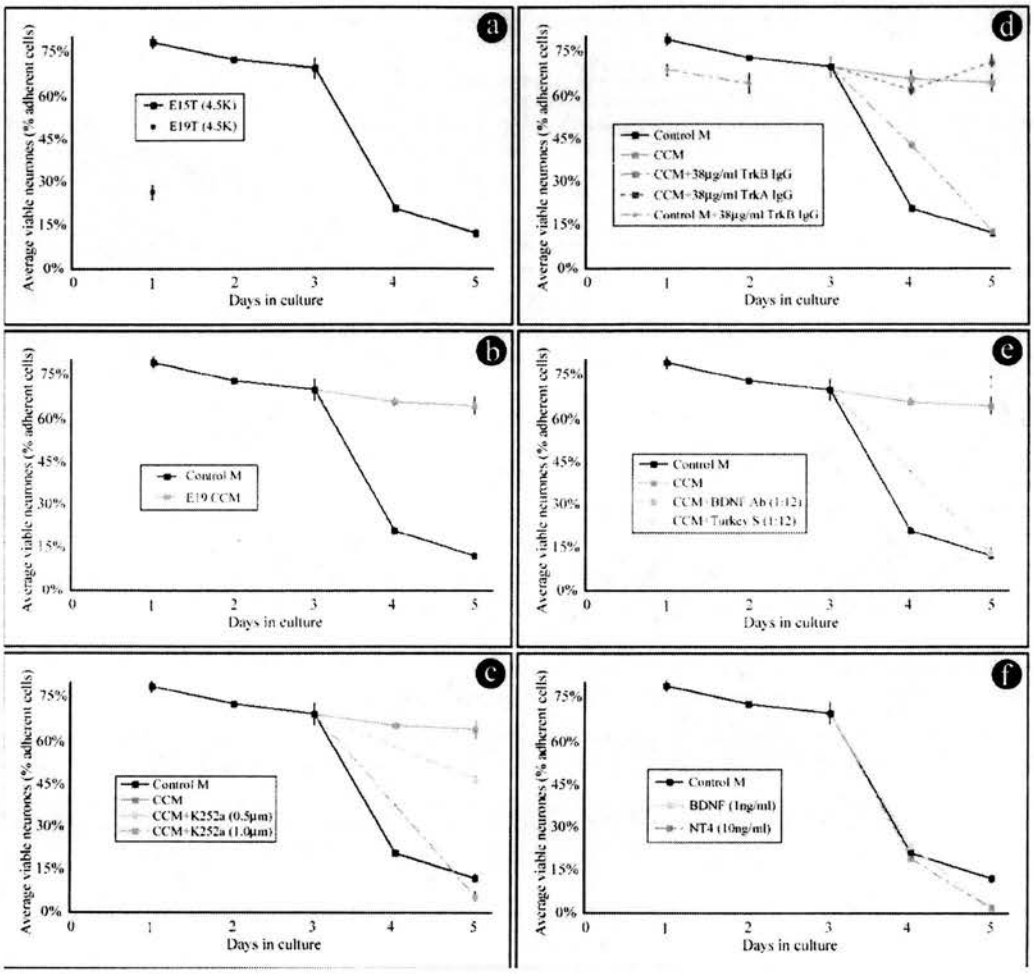
(b) E15 thalamic viability with E19 cortical conditioned medium (CCM): the cells are rescued by the conditioned medium after 5 days in culture.

(c) E15 thalamic viability in the presence of CCM and tyrosine kinase receptor blockers (K252a): K252a blocks the trophic effect of CCM in dose dependent manner.

(d) E15 thalamic viability in the presence of CCM and specific TrkA or TrkB IgGs: survival of thalamic neurons in CCM is sensitive to high concentration of TrkB IgG.

(e) E15 thalamic viability in the presence of CCM and BDNF antibody in turkey serum: anti-BDNF antibody inhibits the effects of CCM on thalamic survival. Note: turkey serum has no effect on thalamic cells in CCM.

(f) E15 thalamic viability in the presence of BDNF or neurotrophin-4: neither neurotrophins can reproduce the CCM effect.


a
Average viable neurones (% adherent cells)
75%
60%
45%
30%
15%
0%
0
1
2
3
4
5
Days in culture
E15T (4.5K)
E19T (4.5K)
d
Control M
CCM
CCM+38µg/ml TrkB IgG
CCM+38µg/ml TrkA IgG
Control M+38µg/ml TrkB IgG
b
Control M
E19 CCM
e
Control M
CCM
CCM+BDNF Ab (1:12)
CCM+Turkey S (1:12)
c
Control M
CCM
CCM+K252a (0.5µm)
CCM+K252a (1.0µm)
f
Control M
BDNF (1ng/ml)
NT4 (10ng/ml)

will be shown in Chapter 4. However, we do not know whether this trophic effect of the CCM was mediated by neurotrophins.

Mechanism by which cortical factors rescue thalamic cells involves neurotrophin signaling

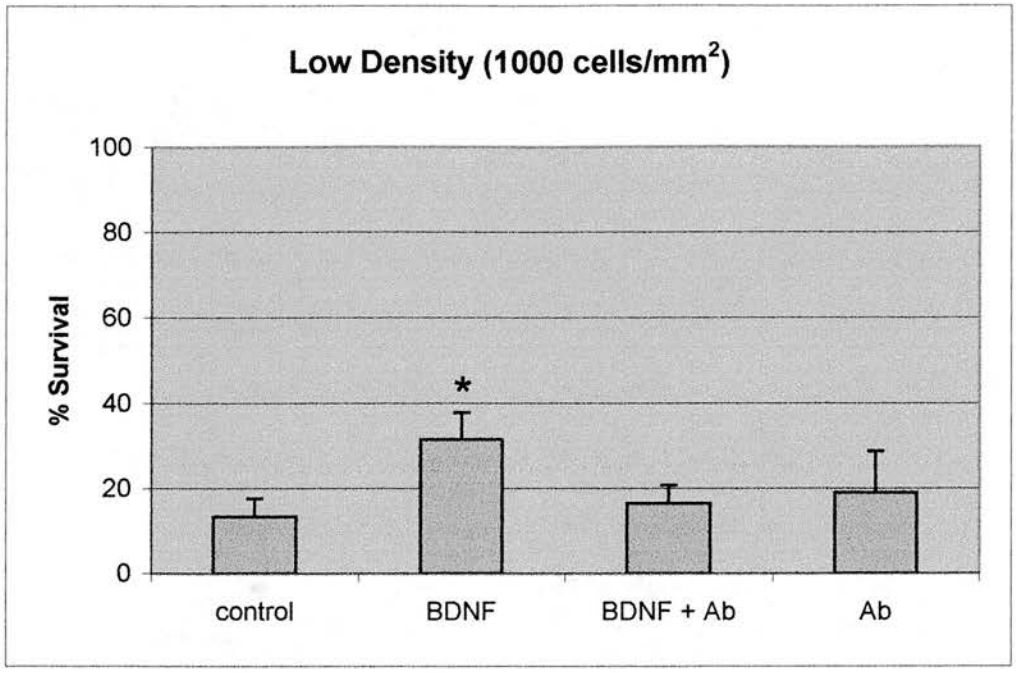
When applying CCM containing 500μM or 1μM K252a (the protein kinase inhibitor specific for neurotrophin signaling) on the third day of culture, the viability assessed two days later was significantly reduced in a dose-dependant manner (Figure 3.6c). This result indicated that one or more neurotrophins in CCM are essential to the thalamic cells. Interestingly, western blot analysis had shown that only BDNF, but not other neurotrophins, was detected in E19 CCM (Dr Leila Vali, unpublished observation). Then we tried to inhibit the activities of either TrkA or TrkB receptors by adding TrkA IgG or TrkB IgG chimeric molecules with CCM on the third day of culture. At 38μg/ml, the concentration shown to have a specific inhibitory effect (Shelton et al., 1995), TrkB IgG inhibited the survival promoting effects of CCM on days 4 and 5 of culture; a low dose, 8μg/ml was not effective (Figure 3.6d). Similar inhibitory effects were found when polyclonal anti-BDNF antibody in turkey serum (1:12 dilution, Ghosh et al., 1994) was added with CCM on the third day of culture (Figure 3.6e). In contrast, TrkA IgG was ineffective even at 38μg /ml (Fig3.6d).

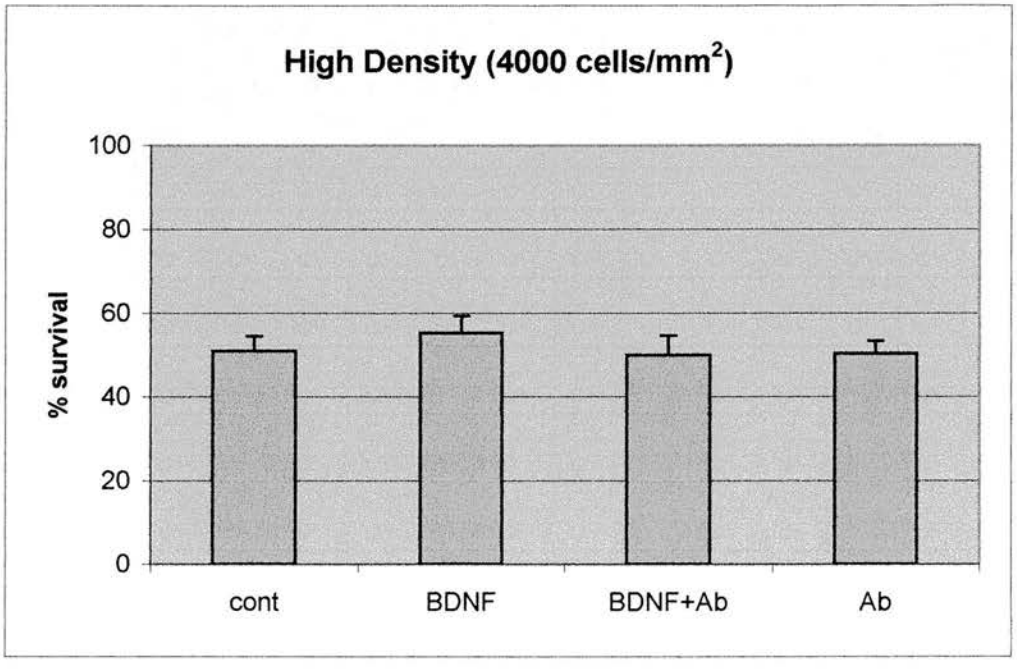
However, at all doses known to be effective in other systems and in thalamic cells at earlier ages (Lotto et al., 1997), BDNF alone failed to maintain thalamic survival after 3 days in culture. The other TrkB ligand NT-4 was also ineffective (Figure 3.6f).

In order to test if BDNF is also required by earlier thalamic neurons, BDNF and anti-BDNF antibody were added to 2-day cultures of low- and high-density thalamic cells. At low plating density (Figure 3.8a), similar to the previous findings (Lotto et al., 1997), BDNF (10ng/ml) promoted thalamic survival, and predictably, the anti-BDNF antibody antagonised the effect of BDNF. But the antibody alone did

Figure 3.8

Graphs showing E15 thalamic viability in 2-day cultures in response to the treatment with BDNF and BDNF antibody. (a) Thalamic viability in low plating density: BDNF significantly promotes thalamic survival. This effect is blocked by the BDNF antibody. The antibody alone does not have an effect on thalamic survival. (b) Thalamic survival in high plating density: thalamic survival is not affected by BDNF, anti-BDNF, or the combination.


Low Density (1000 cells/mm2)
% Survival
100
80
60
40
20
0
*
control
BDNF
BDNF + Ab
Ab


High Density (4000 cells/mm2)
% survival
100
80
60
40
20
0
cont
BDNF
BDNF+Ab
Ab

not have an effect on low-density cells. At high plating density (Figure 3.8b), however, BDNF had no effect on this culture. Anti-BDNF antibody, when applied alone, did not reduce thalamic viability of these cultures. These results indicated that although BDNF can promote thalamic viability at this age in low density, it may not be a major requirement for thalamic cells to survive *in vivo* (as at high plating density, cells can survive even with the anti-BDNF antibody). Moreover, this single application of the antibody suggested that the antibody itself was not toxic at the concentration (1:12) used in this study.

Discussion

1. Significance of *trkB* and *trkC* expression in embryonic thalamus

In support of the *in situ* hybridisation results, TrkB and TrkC have been detected by immunocytochemistry in cultures of E15 thalamic cells (Lotto et al., 1997). Consistent with previous reports in postnatal brain (Ringstedt et al., 1993), *trkB* and *trkC* mRNAs are specifically expressed in several thalamic nuclei of embryos. High levels of *trkB* and *trkC* expression in the anteroventral nucleus suggested that *trkB* and *trkC* regulate limbic system development. Probably with no less importance, both *trkB* and *trkC* may play a major role in the development of many sensory nuclei as they are expressed at a considerable level in those nuclei. At least one sensory region of the thalamus, the dorsal lateral geniculate nucleus, is known to require neurotrophins in development (Ernfors et al., 1992; Cabelli et al., 1995; Cabelli et al., 1997). On the other hand, motor relay nuclei (ventral nuclei) did not express either of the receptor mRNAs. Thus it is unlikely that these two receptors have a role in development of these nuclei (later, I also found that these nuclei have lower cell death at the time of birth compared to other nuclei, suggesting that survival of the neurons in these nuclei may not be under the control of neurotrophins). The correlation between the receptor expression patterns and the distribution of PCD among these nuclei will be discussed below.

2. Evidence that PCD occurs naturally throughout development of the thalamus

2.1. Features and distribution of PCD in developing thalamus

Since the methods used for this study are based on TUNEL labeling and nuclear pyknosis, we can assume that any cells positive by these criteria are dying by apoptosis. I found that many, if not all, dying thalamic cells undergo apoptosis. The distribution of the dead cells within each thalamic nucleus at all ages was found to be

scattered (this is another characteristic of apoptosis, unlike necrotic cells which are often found in clusters, Kerr et al., 1972) (Figure 3.4). The appearance of apoptotic cells was scarce. Similar findings were also illustrated in photomicrographs and drawings in Spreafico et al., (1995) and Waite et al., (1992) (but those studies had insufficient quantitative analysis for the whole thalamus). The estimation of the amount of cell death during development is actually difficult for several reasons. Thalamic cells may undergo PCD via non-apoptotic mechanisms (excluding necrosis) and thus the quantitative methods using TUNEL and pyknotic morphology underestimate the real extent of cell death (Clarke, 1990). Paradoxically, however, there is not enough evidence to date that other types of cell death occur extensively during CNS development (Oppenheim, 1999).

In this chapter, we did not measure the actual number of cell death, but rather the percentage of dead/dying cells over total cells in a specified area (profile count), because the estimation of the number of dead cells is subject to error. To accurately estimate the number of dead cells in each area, they need to be quantified by serial reconstruction (Coggeshall and Lekan, 1996). This is a labor-intensive process and it was not essential to my objective in this study because our main interest was to observe changes in patterns of cell death in thalamic development rather than the number of dead cells.

Regarding cell clearance, changes in the activities of macrophage or microglia may affect the time of clearance of the apoptotic cells. For example, if apoptotic cell clearance by microglia were to be prolonged, we may observed a higher number of apoptotic cells than usual at a particular time. If this happens in the mutants studied, it may cause a misinterpretation in cell counting when I compare the rate of cell death between the mutants and the wild type animals. However, since *trkB* mRNA could not be detected in microglia of normal mice (Frisen et al., 1993), the mutation in functional trkB is unlikely to directly affect microglial function in cell clearance. Thus the increased percentages of dead cells found in this mutant is likely to be due to a real increase in cell death rather than a delay in cell clearance. TrkC, on the other hand, is thought to have a physiological role in glial proliferation

and differentiation and its mRNA can be detected in many glial populations (Kumar and de Vellis, 1996; Kumar et al., 1998; 1999; Barres et al., 1994; Engel and Wolswijk, 1996). However, it is not known whether TrkC directly functions on cell clearance by microglia. And since I found no obvious significant change in cell death in these animals, I shall have no further discussion about this issue.

In vitro quantifications are less problematic since the population of cells in culture is in a limited area and is two-dimensional. Providing that plated cells distribute equally, the ratio between dead cells and the total cell number reflects directly the viability of the cultured population (Coggeshall and Lekan, 1996; Coligan et al., 1995). As mention earlier on the subject of cell clearance, the percentages of cells that die under various conditions *in vitro*, where dead cells are not subject to clearance, did not necessarily correspond with the percentages of TUNEL-positive cells *in vivo*, where dead cells are rapidly cleared.

2.2. Time and phases of the occurrence of PCD in the thalamus

As the cell density falls and the size of the thalamus increases in postnatal development, the percentages of apoptotic cells increase up to 6 times (from 0.2 to 1.2 percent). This suggests that physiological cell death may contribute to the cell density regulation and may shape the brain's morphology (Merry and Korsemeyer, 1997; Oppenheim, 1991). We found that there are at least 2 stages of cell death: a) early on, dying cells are randomly distributed, while (b) later, dying cells are present in some specific nuclei more than other. These two stages of cell death indicate at least two different physiological roles: 1) cell removal by the control for homeostasis during proliferation and 2) cell death by competition for neurotrophic factors.

a) Cell death in the neuroproliferative period of the thalamus

The cell death that occurs in the early stages of development could not be explained by a target-dependent mechanism. This is because it occurs when thalamocortical fibers have not completely formed and thalamic cells are still highly proliferating. Similar patterns of cell death can be found in the early phase of cell

death in spinal cord when cells are highly proliferative and the spinal cord is differentiating the ventral and the dorsal spinal regions (Oppeheim et al., 1999). Apoptosis at this stage can be found preferentially in mitotically active precursor cells rather than in postmitotic cells (Homma et al., 1994).

Previous studies have shown that massive cell death occurs in all neuroproliferative regions in the CNS and have suggested that the majority of cells that undergo cell death are proliferating cells (Blaschke et al., 1996; 1998). In particular in the thalamus, it has been reported that up to 40% of proliferating cells undergo PCD (Blaschke et al., 1998). However, my findings seem to oppose these observations, partly because of the different technique used to measure apoptosis (they used a technique called TUNEL+ which was claimed to be more sensitive than original TUNEL but I found that the increased sensitivity might be due to non-specific staining). In this study, I found that the quantity of cell death in this period is much less than the substantial cell death at the late stage of development where more cells are postmitotic and are innervating their cortical target (see results). The significance of and the mechanism underlying this death among proliferative cells are unknown, but it is likely to be different from cell death that occurs among mature neurons making connections with their synaptic targets.

b) Cell death occurring during cortical innervation

Most thalamic nuclei show a marked increase in cell death around the same time, in the early postnatal period (P1), which makes the rate of thalamic cell death as a whole rise. This increase in cell death appears in distinct, reproducible patterns, which occurred in a stereotypic manner at specific times in development. This therefore represents the normally occurring PCD that is an essential part of embryonic development. In particular, the striking results obtained in the ventral posterior nuclei are similar to those reported in a previous study by Waite and colleagues (Waite et al., 1992). They found that the nuclei reached a peak of cell death in the perinatal period (P0-P2 in rat, compared to our findings at P1 in mouse) and then the rate of cell death declined soon after. In the study of the visual relay nucleus, the dorsal geniculate, the peak of cell death corresponded with target

innervation and the start period of the target dependency (Finlay and Pallas, 1989), which was around E17-P2 (Lotto, 1994).

Although each thalamic nucleus is born at a different time, the increase in cell death in all thalamic nuclei appears to be around the time of target innervation. This suggests that death of cells is not predetermined by the time of their birth or the differentiation of the cell itself but likely to be regulated by the influence of environment at the time of innervation. The most probable factor is the limited supply of target-derived trophic support. Nevertheless, these rises in cell death in all thalamic nuclei at this period occur at different rates. This implies that regulation of cell death in each individual thalamic nucleus is probably independent from that in the others (e.g. they might be controlled by different neurotrophic factors), and/or is determined by differences in their target fields/cell numbers that these nuclei are responsible for innervating (Finlay and Pallas, 1989; Spreafico et al., 1995; Oppenheim, 1999).

3. The roles of neurotrophins and their receptors in regulating thalamic PCD

3.1. *In vivo*: PCD in trkB and trkC mutant thalamus

TrkB, but not TrkC, regulates cell death in thalamus

Although *trkB* and *trkC* mRNAs were expressed in overlapping patterns in many regions of the thalamus, studies of cell death in mice lacking one of these functional receptors revealed different results. There was increased cell death in the *trkB* mutant. To rule out a non-specific cell death due to secondary consequences of the mutation, TrkC mutant mice can be used as a control as they also have one functional neurotrophin receptor disrupted but show no significant increase in cell death in late gestation when compared with wild types. In E19 *trkB* knockout mice, increases in cell death were found in all thalamic nuclei that would normally express trkB, i.e. AVT, VP, and DLG). This evidence strongly suggests that TrkB functions as a survival regulating molecule in these thalamic nuclei at this late stage of

development. In contrast, despite a high level of expression of *trkC* in thalamus, there is no difference in thalamic cell death in *trkC* mutants. Thus it is possible that *trkC* might play other role(s) than being a survival receptor for the thalamus. Lewin and Barde (1996) suggested that NT-3 could regulate neuronal number by influencing the generation of neurons from precursor, via its mitogenic effects and stimulation of cell cycle exit. In order to investigate this issue properly, I have conducted a BrdU proliferation study in both cortex and thalamus of *trkC* mutant mice as described in Chapter 7.

In fact, the increase in total cell death in the whole thalamus (in particular in DLG) of the *trkB* mutant appeared not only at E19 but was found as early as E17 and extended until the time of birth (P1). This period is also known to be the time of target innervation (Catalano et al., 1991; Ferrer et al., 1992; Lund and Mustari, 1977). This increase in cell death in the *trkB* mutant thalamus, however, precedes the natural peak of cell death in normal thalamus (which occurs around P1). This means that many thalamic cells begin to require TrkB signaling for survival well before the natural period of cell elimination.

Although there was a dramatic rise in cell death in VP at P1 and this area would normally express *trkB*, there was no increase in cell death in VP of the *trkB* mutant at this age as I initially expected. There is no obvious explanation for this but it is possible that either 1) VP cells of the mutant that require TrkB signaling die before the peak of normal PCD is reached (at E19, before P1) or 2) dead cells at P1 might be a different population of cells which does not require TrkB signaling.

Despite the fact that the percentages of cell death in mice lacking tyrosine kinase domain of the TrkB is higher than the wild type thalamus, the differences in the percentages between the two are seemingly low. The pattern of cell death during the peak time of the thalamic PCD (particularly at P1) is not much affected by the mutation. Thus there may be other mechanisms than TrkB kinase signaling involved in regulating thalamic PCD, in particular, in the areas that do not normally express *trkB*. The examples for such mechanisms include: 1) compensatory effects by other

neurotrophins (either the known or unknown factors), 2) signaling of TrkB ligands (BDNF & NT-4) via non-catalytic domain of the tyrosine kinase receptor or p75 to keep thalamic cells survived, 3) maintenance of thalamic survival by non-target-derived mechanisms such as afferent fibers, and cellular interactions (e.g. regulation by autocrine or cell-cell contact mechanisms) (Oppenheim 1991, Magowan and Price 1996, Lotto et al., 1997).

Regarding the possibility that the increase in cell death found in the *trkB* mutant is due to signaling compensation via other kinase receptors, it is likely that *trkC* is the candidate for this compensation. This is because both *trkB* and *trkC* are often expressed in overlapping patterns in the developing thalamus. Double mutation of these two receptor genes may help to gain a better understanding of their role in controlling thalamic PCD. Indeed previous studies have shown that double *trkB-/-; trkC-/-* knockouts showed severe sensory deficits. However, their studies did not show any significant defect in CNS structure (Silos-Santiago et al., 1997).

In DLG, although we found that there is an increase in cell death in the *trkB* mutant, it has also been reported that TrkB binding ligands regulate synaptic plasticity in later stages of visual development (review by McAllister et al., 1999). Injection of NT-4 can rescue lateral geniculate nucleus cells from the effects of monocular deprivation (Riddle et al., 1995). This rescue effect in late postnatal animals was only to bring back shrunken cell bodies to their normal size, and does not involve the prevention of apoptosis. Together with my data, there is strong evidence to believe that TrkB plays more than one role in thalamic development, first a survival function, and later in plasticity.

Considering the possible role of the putative TrkB ligands, BDNF and NT-4, in regulating thalamic cell death, the expressions of both neurotrophins are different and dynamic during development. Both BDNF and NT-4 are produced at early embryonic ages. BDNF is produced early (E9) and continues to increase postnatally until the first postnatal week (Dugich-Djordjevic et al., 1992; Friedman et al., 1991). In contrast, the level of NT-4 is maximal in the midgestation period (E15) and

gradually declines with the lowest level being around birth. Since the times of expression are different, it is possible that thalamic nuclei, especially DLG, might require these neurotrophins at different time points and these neurotrophins therefore regulate the time of PCD. For example, in early age, NT-4 may be a major trophic molecule which transiently supports DLG neurons (as it is highly expressed in early age), while trophic molecules in the young cells might be redundant. When the cells mature and the availability of other neurotrophins is more limited, BDNF becomes the major source of neurotrophin (as the BDNF production is increased around E15-E17). These cells may become more restrictive and require only BDNF molecules in later stages.

3.2. *In vitro*: BDNF is required for the late embryonic thalamic cells

Early thalamic cells (at E15) are promiscuous and respond to several neurotrophic factors including neurotrophins and fibroblast growth factors (Lotto et al., 1997). However, single application of several neurotrophins failed to rescue thalamic cells at the older ages (see Results). Thus the maturing thalamic cells may need more than one trophic factor and/or need other neurotrophic factors to survive.

Diffusible factors from cerebral cortex are known to promote growth and survival of thalamic cells (Hisanaga and Sharp, 1990; Lotto and Price, 1995; Bolz et al., 1990). But the factors involved have not been successfully identified. One neurotrophin, BDNF, has been found to be a component of the cortical conditioned medium (L. Vali, unpublished observation). Further evidence supports the possibility that late thalamic cells require BDNF comes from the observation that K252a, TrkB IgG, and anti-BDNF antibody successfully block the effects of the cortical conditioned medium, whereas TrkA IgG could not. This also corresponds with our *in vivo* data on TrkB mutant. Nevertheless, BDNF or any neurotrophin alone cannot support thalamic cells older than E17.

Collectively, our results suggested the following. 1) Before cortical innervation, thalamic cells may have sufficient trophic support because of their promiscuity. 2) Thalamic cells may narrow their responsiveness around the time of

cortical innervation, and become dependent on trophic molecules that are released from the target. 3) Thalamic survival is regulated partly by a neurotrophin – BDNF (via TrkB signaling) but that BDNF alone is not sufficient for the mature thalamic cells.

4. Summary of possible mechanisms regulating PCD in the thalamus and some unanwered questions

This study has determined the period of perinatal thalamic PCD and has indicated that survival of thalamic cells is regulated by neurotrophic factors, in particular, BDNF and TrkB signaling. But there may still possibly be other factors involved in the regulation of thalamic PCD. Moreover, the underlying cause(s) making thalamic cells come under trophic factor regulation and undergo PCD at a specific perinatal period is unknown. Endogenous factors such as Bcl-2 may be involved in the timing of this event (see Chapter 4 for detailed discussion, Greenlund et al., 1995; Holm and Isacson, 1999). In the perinatal period, it is possible that thalamic cells may undergo trophic switching or may narrowing their trophic dependence to respond only to factors produced by the target (Davies, 1996; Lotto et al., 1997). This was tested as described in the subsequent chapter. In addition, neural activity and connectivity during development might be essential to determine the time of the trophic dependency in thalamic cells (Magowan and Price, 1996).

Although the period of the thalamic PCD coincides with the period of thalamocortical innervation, the correlation between the two is difficult to prove. However, several observations indicated that competition for trophic factor among thalamic cells (according to the Neurotrophic Hypothesis) is likely to occur. In Chapter 6 I have shown that thalamic cortical fibers might be essential for thalamic cells to obtain sufficient trophic factors.

Chapter 4: Neurotrophic effects of condition media in dissociated thalamic cell culture

Introduction

In previous chapters, I showed that in cultures of dissociated cells, the survival of developing thalamus is promoted by increasing their cell density (see Chapter 2). This can be regarded as an intrinsic property of the thalamus. However, the high cellular density of E15 thalamic cells could not maintain the viability of cells when they were cultured for 3-5 days (which corresponds to the period of innervation and the time of birth *in vivo*). Neither could high cellular density maintain the viability of cells taken from the perinatal age. Lotto and I also showed that the survival of developing thalamic cells is increased when cells are supplied with external trophic support, culture medium conditioned with E19 cortex (see Chapter 3, *in vitro* experiments). Although at least one neurotrophin (BDNF) is present in this conditioned medium and Trk signaling (TrkB) is known now to be essential, it is not sufficient as a sole factor for thalamic survival. Some unidentified-neurotrophic factors released from conditioned medium might also play a crucial role in regulating thalamic survival.

In this chapter, I describe my investigations focused on the basic properties of conditioned medium in thalamic cell cultures with less concern for any particular neurotrophins or trophic factor families. Diffusible factors from various tissues as well as from the cerebral cortex have been shown to promote both survival and neurite outgrowth of thalamic neurons of different ages (Hisanaga and Sharp, 1990; Lotto et al., 1997; Lotto and Price, 1994). For example, cortex, cerebellum, medulla, and thalamus can promote neurite growth from E16 thalamic explants in culture (Rennie et al., 1994). Co-culturing with different ages of cortical explants also varies the amount of neurite outgrowth from thalamic explants. Although trophic factors released from some of these different tissues may have similar survival or growth promoting effects, the molecules responsible for both effects may be different. My

interest was to test the ability and specificity of these unknown molecules from different tissues at different ages in promoting thalamic survival.

First I confirmed the basic properties of the conditioned medium by testing the effect the withdrawal of E19 cortical condition medium. This is to verify that the conditioned medium has a real trophic role (i.e. that it maintains thalamic cell viability) rather than merely aiding initial recovery from dissociation. Many trophic substances released from conditioned medium or from nervous tissue extract which promote neuronal populations are often temperature sensitive and are proteinaceous (Oh, 1976). In parallel with the antibody experiment showing the likelihood of neurotrophin involvement in the previous chapter, I confirmed that the trophic effects in the conditioned medium are from proteinaceous or temperature sensitive substances. This was done by heat treating the conditioned medium.

If the notion of the neurotrophic hypothesis applies to the thalamus, we may predict that thalamic cells at the time of cortical innervation should become dependent on their major target – the cerebral cortex. I tested if these thalamic cells respond specifically to target (cortical)-derived factors. This was done by comparing the trophic effects of various media preconditioned with different tissues including the thalamus itself on dissociated young thalamic cells cultured until the age at which they would have innervated cortex.

As reported by others, some neuronal populations also undergo a neurotrophic switch during the target innervation period i.e. those neurons which formerly respond to one or more trophic factor(s) become dependent on another factor(s) released from the target (see review in Chapter 1; Birren et al., 1993; Davies, 1988a; b; Davies, 1994a; Pinon et al., 1996). If this switch occurs in the thalamus, I predicted that maturing thalamic cells should preferentially switch their responsiveness to depend almost exclusively on trophic factors derived from cortical tissue and no longer respond to factors produced intrinsically in thalamus of the same age.

Although thalamic neurons maintain their own survival before the time of cortical innervation, it is not known how this ability declines with time. I tested if trophic factors released from the thalamus itself (intrinsic factors), as well as from the cortex of different stages (using E15, E19, and P2 tissues) produce similar trophic effects on later thalamic neurons. However, it is difficult to obtain trophic factors released from the maturing thalamus (> E19) in conditioning medium as the thalamus at this age does not survive well. Since several studies suggested that unidentified trophic factors from neural tissue extract promote viability in various types of nervous tissue (Oh, 1976; Yin et al., 1992; Karasek et al., 1977), first I tried to obtain trophic factors directly by this tissue extraction method. However, the attempt failed but I saw some increased trophic effects of maturing thalamus by adding K^+ to maintain its survival, as it has been shown to increase neuronal depolarisation and activity (Magowan and Price, 1996; Lipscombe et al., 1988).

Embryonic thalamic neurons in cultures are heterogeneous, as *in vivo*. The thalamus contains 2 main types of neuronal population 1) interneurons and 2) projecting neurons. The major neurons of each subpopulation are 1) GABAergic interneurons (neurons producing gamma-aminobutyric acid as a neurotransmitter) and 2) glutamatergic projecting neurons (neurons producing glutamate as a neurotransmitter) (Jones, 1985). Both GABAergic neurons and glutamatergic neurons in other brain regions have been shown to be affected by BDNF or NT-4/5 in many aspects including differentiation, maturation, electrochemical activity, and induction of the production of neurotransmitters (Huang et al., 1999; Mizuno et al., 1994; Widmer and Hefti, 1994a; 1994b). Moreover, some neurotrophic factors can both induce differentiation and selectively promote survival of certain neuronal subpopulations at the same time (Hisanaga and Sharp, 1990; Huang et al., 1999; Mizuno et al., 1994; Widmer and Hefti, 1994). It is thus possible that the trophic effects of different sources of conditioned medium selectively rescue different subpopulations of thalamic neurons.

Here I tested the possibility that 1) conditioned media from thalamus and cortex of different ages has different effects on GABAergic interneuron

subpopulations, and 2) the cortical conditioned medium selectively promotes survival of non-GABAergic neurons (presumably mostly projecting neurons) and shares a similar effect with BDNF and NT-4/5.

Materials and Methods

Animal/Tissue preparation

C3H mice were obtained from overnight matings. Animals at E15, E19 and P2 were sacrificed. For dissociated cell cultures, E15 thalami were removed as described in Chapter 2 and dissociated. For conditioning media and preparing tissue extracts, E15, E19, and P2 thalami, E15, E19, and P2 cerebral cortices, E19 tecta, E19 cerebella, E19 livers, and E19 hearts were removed from foetuses and placed in an adequate volume of culture medium.

Culture methods

Detailed methods for cell dissociation, culture processes, and calculation of plating density were described in detail in Chapter 2. In this Chapter, tissue culture with cells plated at 1,000 cells/mm^2, and 4,000 cells/mm^2 were regarded as low density and high density respectively. After 24h in culture, 80µl of culture medium in some samples was replaced by fresh culture medium, conditioned medium, or tissue extracts in culture medium, depending on the experiments. Withdrawal experiments were done by removing E19 cortical conditioned medium after 72h (3 days), and 120h (5 days) and replacing by the same volume of fresh culture medium.

Conditioning medium

Explants of thalamus, cortex, cerebellum, tectum, liver, and heart aged from E15 to P2 were sliced at 500 x 500 µm^2 with a McIlwain tissue chopper. Chunks of the explant were put in culture medium (serum-free). 40 pieces of each type of explants in 100µl culture medium were put on a 24 well Transwell collagen culture plate (Costar, Transwell-COL) which was suspended over wells filled with 400µl culture medium. The explants were cultured for 24h and the Transwells containing explants were removed. Only the remaining culture medium in the lower part, which was now conditioned, was stored at -70°C. Before these frozen conditioned media

were added to the dissociated cell culture, they were thawed in a 37°C incubator for at least 1h. E19 thalamic and E19 cortical explants were reserved, fixed with 4% paraformaldehyde, and histological processed (sectioned at 10μm), to determine their viability.

For control cultures that had been left for longer than 24h, most control culture medium was not replaced. This is because replacing old culture medium with fresh or normal culture medium, which had been preincubated with collagen Transwell filters, did not rescue thalamic cells after 3 days in culture.

Heat treating of conditioned medium

E19 cortical conditioned medium was heated to 85°C for either 3 minutes or 30 minutes before it was added to 24h thalamic cell cultures. The viability of samples with added preheated normal culture medium was compared with the viability of samples with normal culture medium that had no heat pretreatment.

Tissue extracts in culture medium (homoginisation experiment)

Explants of thalamus, cortex, cerebellum, tectum, liver, and heart aged E15 or E19 were sliced at 500 x 500 μm^2 with a McIlwain tissue chopper. Every 200 pieces of each type of explants were put in 2.5ml culture medium (serum-free, same proportion as in conditioning medium; 40 pieces/0.5ml). Tissue extracts were prepared by homogenizing the tissue in the culture medium with a glass tissue grinder and centrifuging the homogenate in a Beckman ultracentrifuge at 100,000g 4°C for 2h according to Oh (1976). The supernatant was collected and filtered (0.45μm). These extracts then were added to the dissociated cells that had been in culture for 24h.

In the experiment testing the toxic effects of the extracts, these extracts were also diluted 1:10, 1:100, and 1:1000 in culture medium before adding to the dissociated cells that had been in culture for 24h. Cells were fixed after 2 and 5 days.

Direct application of K^+ on dissociated cell culture

In this experiment, after culturing for 24h, 80μl of culture medium was replaced by culture medium containing 0.05mM, 0.5mM, 5mM, or 50mM KCl to test the effect of potassium on thalamic cell cultures. The quantitation of cell viability was done and compared with the control.

Conditioning media with the presence of KCl

For explants that had been used for conditioning media and had had K^+ added, 0.05mM, 0.5mM, 5mM, or 50mM KCl were added to culture medium to conditioning the media. After 24h of conditioning in a 37°C incubator, E19 explants of both thalamus and cortex cultured with different K^+ concentration were fixed, embedded and sectioned at 10μm to determine viability. Culture media conditioned with thalami or with cortices in different concentration of K^+ were taken and stored at -70°C. Before used, these conditioned media were thawed and prewarmed at 37°C.

Immunohistochemistry for GABA

Dissociated thalamic cells that had been fixed in 4% paraformaldehyde at room temperature for 15 minutes were washed with 3 times 2.0% Triton-X in PBS for 5 minutes each. Cells were then preblocked with normal goat serum (NGS) in TBS (1:4 NGS:TBS) for 10 minutes. Cells were incubated with affinity purified (polyclonal) rabbit anti-GABA antibody (1:1000, Sigma) in 4°C overnight. The following day, cells were washed twice in PBS for 5 minutes each. A second preblocking with NRS followed. The sections were next treated with a biotinylated secondary antibody (goat anti-rabbit, 5μl/ml) at 4°C overnight. When this incubation was complete, the cells were once again washed with TBS, then treated with avidin and biotinylated complex kit (ABC kit, Vector) according to the manufacturer's instructions for 30 minutes. Then cells were washed and reacted with 3,3'-diaminobezidine (DAB, Sigma) solution (0.05% DAB, 750μl tris buffer, 20μl 0.1% H_2O_2 and one flake of imidazole) for 8 minutes.

Before proceeding with the investigation, I optimised the concentration of the primary antibody (the anti-GABA antibody) by dilution studies. The 1:1000 was the lowest concentration that gives the best labeling and therefore was used in this study. The immunoreactive cells were classified into two classes; heavy and lightly labeled cells. For secondary antibody, the 1:200 dilution was used as a routine concentration in our lab. For negative controls, the reactions were performed with the omission of primary antibody. Since the antibody is polyclonal, non-specific background staining may occur (Harlow and Lane 1988). I tested if at the dilution of 1:1000 the antibody had such non-specific staining. In one reaction (among the first run of the experiments), the antibody was preblocked with GABA molecules (1:100, mol/mol) (so called preadsorbed antibody) before adding to the cells. These samples were compared with antibody-omitted negative controls and the experimental sample without preadsorption. No non-specific staining was found in the sample with preadsorbed antibody.

Samples of GABA reactions and the negative control are shown in Figure 4.8.

Analysis

Cell viability analysis in culture

All quantitative measurements were done with Hoechst staining as detailed in the previous chapter. Data for all viability experiments were analysed and their significance was determined with Student's t-test (comparing with control), and by ANOVA if more than two experimental treatments were compared.

Cell viability analysis in explants used for conditioned medium

To assess the viability of organotypic thalamic explants that had been used for conditioning medium, explants of each age tested (E15 and E19) were fixed, embedded in wax, sectioned at 10μl, and stained with 5μg/ml Hoechst. For five to ten randomly selected explants of each tissue, the viability was assessed by counting

the proportion of living cells and total cell number in a grid square under a UV filter of a flurescence microscope.

Data for all viability experiments (both dissociated cultures and explants) were analysed and their significance was determined with Student's t-test.

Quantification for GABA immunocytochemistry

Only 'living cells' that were reactive to GABA antibody were counted as GABA positive cells (because GABA positive dead cells were difficult to identify as they may be confused with cell debris or artifacts). GABA positive cells were divided into 2 groups; highly labeled cells and lightly labeled cells. Counts were made as percentages of the heavily labeled cells and the lightly labeled cells relative to the total living cells (Figure 4.9).

I was unable to perform GABA reactions using fluorescence immunocytochemistry. Cell counts with Hoechst according to previous cell viability criteria cannot be applied in light microscope. Live cells in this experiment were determined as the phase dark healthy-looking cells producing neurites.

The quantitation of GABA positive cells was tested by analysis of variance (ANOVA) instead of Student's t-test. This is because I compared also the difference among different treatment groups in addition to comparing each experimental groups with control.

Summary of the n-number for the experiment in this chapter

Experiment	mice used in conditioning/ extracts	mice used in dissociation	wells	no. of culture
1. Conditioned medium withdrawal	1	3		10 in all of each group
2. Heat treatment	2	2		3 in all groups
except: cells in 5 days *in vitro* (DIV) with CCM,				n = 4
cells in 5 DIV with 85°C 30' CCM,				n = 5
3. Low density with conditioned medium	2	1		5 each
4. High density with conditioned medium	5	4		
2 DIV				n = 10 each
5 DIV with e15thcm, e15ccm, e19thcm, e19ccm				n = 15 each
5 DIV with e19cbcm, e19tecm, e19heart, e19liver				n = 10 each
5. Extract experiment	4	6		n = 4 each
6. Conditioned medium with K^+	4	4		n = 4 each
7. GABA experiment	4	4		n = 4 each

Results

1. Withdrawal of cortical conditioned medium

Dissociated E15 thalamic cells at high plating density progressively died over a period of 6 days *in vitro* (DIV) but around 50% or more of cells survived up until 3 DIV. By the time that corresponded to the period of target innervation *in vivo* (from E18 *in vivo* or cells > 3 days DIV onwards), the majority of cells did not survive. For example at 6 DIV the viability was only 1.5 $\pm$ 1.95 %, unless cells were provided with the medium preconditioned with the E19 cerebral cortex (CCM) after the first day of culture (viability = 52.03 $\pm$ 6.58 % at 6 DIV) (Figure 4.1, 4.2). This trophic effect was abolished if the CCM was removed and replaced by normal culture medium. When CCM was withdrawn after 3 days in culture, thalamic cells did not survive at 6 DIV (viability = 5.05 $\pm$ 0.4%). Interestingly, if CCM was maintained until the day before the end of culture period (6 DIV), this withdrawal did not completely abolish the viability. The thalamic viability in this case was 36.02 $\pm$ 1.95% ($p < 0.05$ comparing with 1 DIV culture).

2. Heat inactivation of cortical conditioned medium

In this set of experiments, thalamic viability in all samples was generally lower than other sets of experiments (viability was below 40% after 2 DIV). I do not know the reason but it is possibly due to errors during technical-preparation procedures. When heated at 85°C for as long as 30 minutes, normal culture medium (CM) was still able to provide essential substances required by the dissociated thalamic neurons at 2 DIV (Figure 4.3, Table A). The level of survival promoting effects in the heated culture medium was not significantly different compared with that in the non-heated control (heated: 38.23 $\pm$ 5.86 %, control: 37.02 $\pm$ 1.65 %). Similarly, heat treatment had no effects in the cultures with cortical conditioned medium (CCM) in 2 DIV cultures. 2 DIV thalamic neurons with a supply of preheated CCM or non-heated CCM survived normally (38.86 $\pm$ 5.39 %, and 39.06 $\pm$ 2.32 % respectively). This suggested that heat inactivation does not degrade

Figure 4.1

Photomicrographs of thalamic cells cultured for 5 days (high plating density), stained with 5μg/ml Hoechst and viewed under a UV-filter on a fluorescence microscope.

(a) With normal culture medium (CM), most thalamic cells die with pyknotic morphology (red arrows).

(b) With cortical conditioned medium (CCM), many cells remain healthy with large nuclei (green arrows).

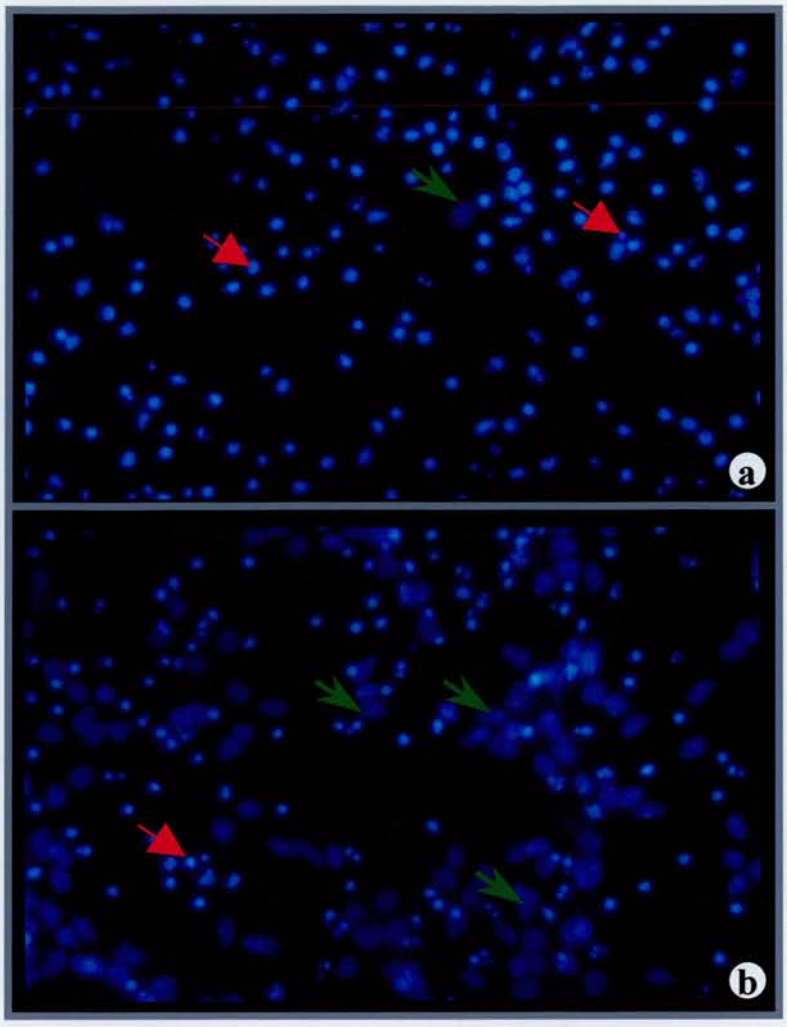

a
b
100μm

Figure 4.2

Graph showing the withdrawal effects of cortical conditioned medium (CCM) on the survival in high-density thalamic cultures. Survival of thalamic cells in normal medium dropped fairly steadily up to 5 days in culture, with the sharpest declination at 3 days (blue). CCM rescued over 50% of thalamic cells after 5 days in culture (pink). When CCM is withdrawn on the 3rd, most cells do not survive in 5 day-culture (orange dotted line). When CCM is withdrawn on the 5th day, survival percentage of thalamic cells is marginally decreased (light blue).

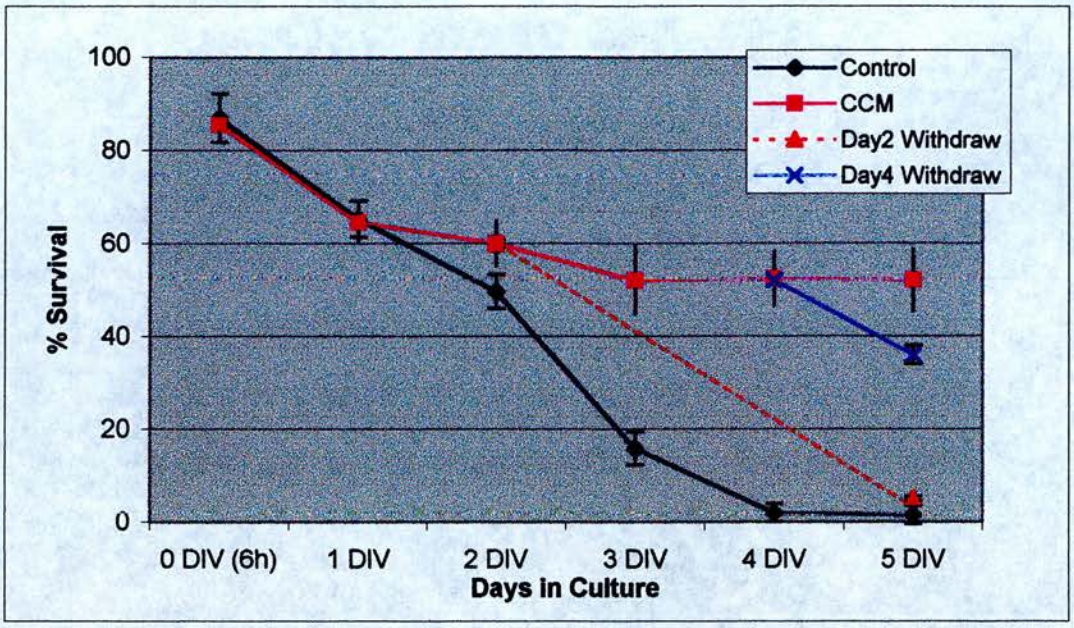

100
80
60
40
20
0
% Survival
Control
CCM
Day2 Withdraw
Day4 Withdraw
0 DIV (6h)
1 DIV
2 DIV
3 DIV
4 DIV
5 DIV
Days in Culture

Figure 4.3

Tables showing the trophic effects of cortical conditioned medium (CCM) and normal culture medium (CM) with heat pretreatment (85°C) on the survival of dissociated thalamic cells at high plating density.

(a) In 2-day cultures, survival of thalamic cells in either CM or CCM that has been preheated for 3 or 30 minutes is not different from thalamic cells cultured with fresh CM or CCM.

(b) In 5-day cultures, most thalamic cells in CM die while CCM promotes thalamic survival. However, 30-minute-preheated CCM has much less trophic effect than the non-heated CCM.

85°C Heat inactivation

Table A

2 DIV	% Survival		
	without heat	3 minutes	30 minutes
CM	37.02 ± 1.65%	37.37 ± 2.48%	38.23 ± 5.86%
E19CCM	38.86 ± 5.4%	35.73 ± 6.02%	39.06 ± 2.32%

Table B

5 DIV	% Survival		
	without heat	3 minutes	30 minutes
CM	0.39 ± 0.36%	0.39 ± 0.39%	0.31 ± 0.27%
E19CCM	39.34 ± 1.96%	40.82 ± 3.14%	20.29 ± 2.98%* ($p < 0.05$)

molecules present in the normal culture medium within the designed culture period. Thus, if heat treatment altered the trophic effect of CCM at later stages, it would be likely that heat treatment reduces the additional trophic molecules rather than reduces the levels of essential molecules already existing in culture medium before conditioning.

When cultured for 5 days the cells did not survive either with the heated or non-heated normal culture medium (Figure 4.3, Table B). Cortical conditioned medium maintained the viability of thalamic cells at the stage. But when it was heated for half an hour, survival of thalamic neurons decreased by half of that seen in the non-heated CCM (20.29 $\pm$ 2.98 %, compared with unheated 39.34 $\pm$ 1.96 %, $p < 0.05$). Thus, the trophic effect of the cortical conditioned medium is heat sensitive. However, heat treatment for 3 minutes did not reduce the trophic effect of CCM.

3. Effects of various sources of conditioned medium

3.1. Effects on E15 thalamic neurons at high plating density

At high plating density, around 50% or more thalamic cells remained viable after 2 days in culture. The viability of these cells was not increased in the presence of any conditioned medium of any tissue explants studied (Figure 4.4a).

At 5 DIV (equivalent to P0 *in vivo*), E15 thalamic neurons, which would normally die, did respond to conditioned media obtained from many tissue types (Figure 4.4b, c). All of the effects were only found in the conditioned medium from neuronal sources in varying degree. Conditioned media from heart and liver did not enhance thalamic survival (Figure 4.4c). E19 cerebellar conditioned medium (CBCM, non-target conditioned medium) promoted thalamic survival at the same level as E19CCM (effects of E19CBCM = 50.42 $\pm$ 4.7%, E19CCM = 50.53 $\pm$3.43 %). This was in contrast to the previous finding that cerebellum had less neurite-promoting effects on thalamus than cerebral cortex (Lotto and Price, 1995).

Figure 4.4

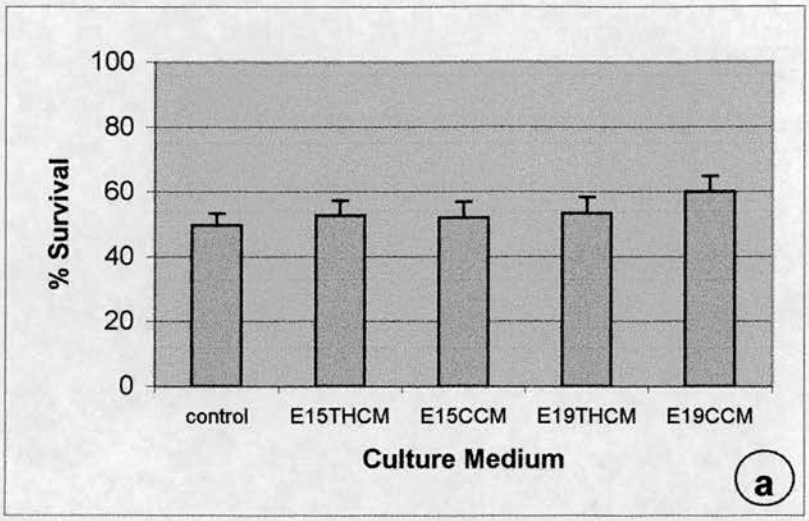
Graphs showing thalamic survival in high-density cultures in response to different conditioned media.

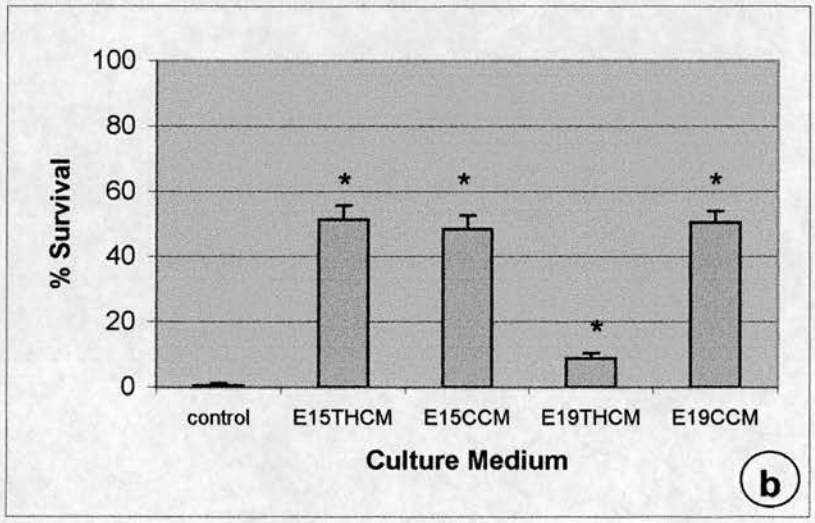
(a) After 2 days in culture, most thalamic cells at high plating density in all types of the given medium. Conditioned media had no survival promoting effects.

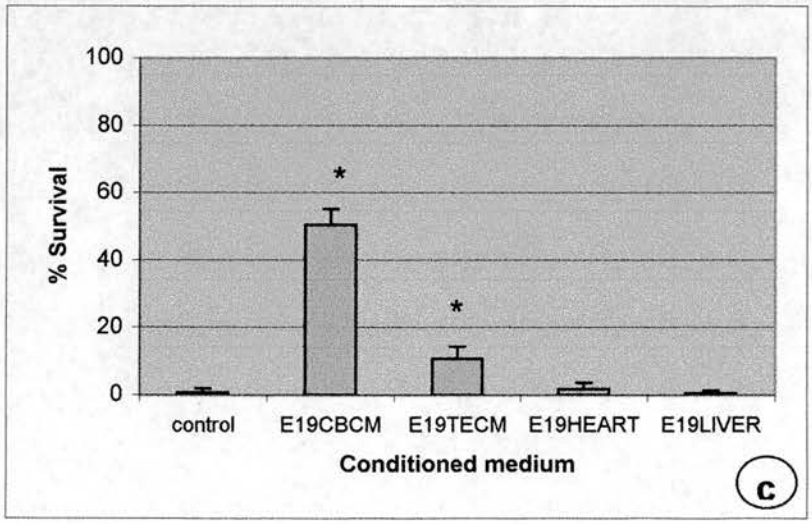
(b) Survival of thalamic cells after 5 days is reduced markedly in control media. Medium conditioned with E15 thalamus, E15 cortex, or E19 cortex restores the viability. However, E19 thalamic conditioned medium rescues only a small number of thalamic cells.

(c) In the presence of medium preconditioned with other tissues (non-target and non-thalamic tissues), they only respond to medium preconditioned with neuronal tissue but not to medium conditioned with heart or liver. E19 cerebellar conditioned medium has a similar trophic effect to E15THCM, E15CCM and E19CCM. Tectal conditioned medium produces only a small increase in survival.

Abbreviations: cont: control; E15THCM: E15 thalamic conditioned medium; E15CCM: E15 cortical conditioned medium; E19THCM: E19 thalamic conditioned medium; E19CCM: E19 thalamic conditioned medium. E19CBCM: E19 cerebellar conditioned medium; E19TECM: E19 tectal conditioned medium; E19HEART: medium conditioned with E19 heart; E19LIVER: medium conditioned with E19 liver.


100
80
60
40
20
0
% Survival
control
E15THCM
E15CCM
E19THCM
E19CCM
Culture Medium
a


100
80
60
40
20
0
% Survival
*
*
*
*
control
E15THCM
E15CCM
E19THCM
E19CCM
Culture Medium
b


100
80
60
40
20
0
% Survival
*
*
control
E19CBCM
E19TECM
E19HEART
E19LIVER
Conditioned medium
c

Conditioned media from other neuronal tissues also had trophic effects on the thalamic cells. E19 tectal conditioned medium (E19TECM) and E19 thalamic conditioned medium (E19THCM) significantly promoted thalamic survival (all $p < 0.05$ compared with 5 days control), although to a much lesser extent (10.69 $\pm$ 3.73 % and 8.72 $\pm$ 1.72 % respectively) (Figure 4.4b).

Conditioned medium of tissues of different ages had different effects on thalamic survival. E15CCM and E19CCM had a similar trophic effect. However, when I compared the conditioned medium of E15 thalamus (E15THCM) and E19 thalamus (E19THCM) (viability of E15THCM = 51.28 $\pm$ 4.4 %, E19THCM = 8.72 $\pm$ 1.72 %), E19THCM failed to maintain the high level of survival of the dissociated thalamic cells. This was not surprising as this result does correspond with the reduction in the viability of the dissociated thalamic cells at high density on their own when were cultured for more than 3 days (> E18).

3.2. Effects on E15 thalamic neurons at low plating density

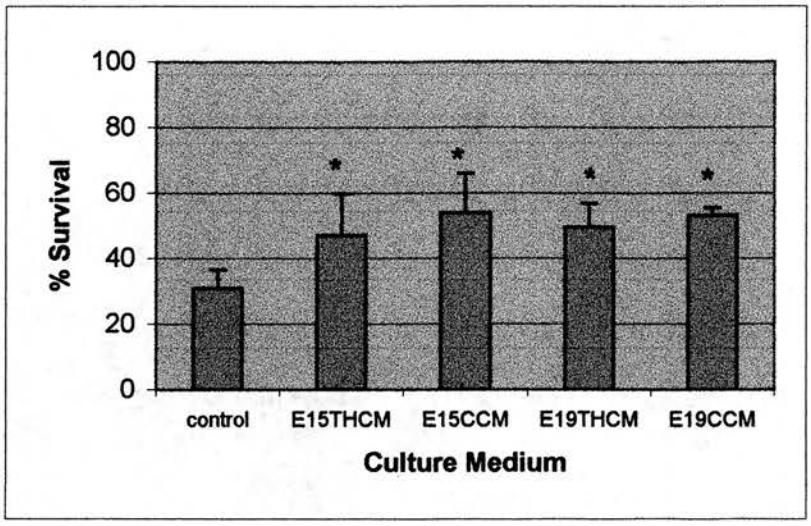
In 2 DIV cultures, thalamic cells taken from E15 embryos survived well at high plating density. As seen in Section 3.1, conditioned media had no trophic effects on high-density thalamic cells. This raises two possibilities that either early thalamic cells do not respond to trophic factors from any conditioned medium or increase in intrinsic trophic molecules and/or cell-cell interaction present in high density is sufficient to maintain the cell viability. To test these possibilities, I lowered the plating density and retested the effects of the conditioned media.

In 2 DIV low-plating density cultures, only 30.88 $\pm$ 5.83 % of the thalamic cells survived. Thalamic cells at this condition were promiscuous to trophic factors from several tissue types. All conditioned media had the same trophic effects on low-density cells (Figure 4.5). No difference in trophic effects was found among the effects of these media ($p > 0.05$). Contrary to the effects on 5 DIV at high density, E19THCM was sufficient to promote thalamic survival of 2 DIV low-plating density cultures at the same level as E15THCM, E15CCM, and E19CCM.

Figure 4.5

Graph showing survival of thalamic cells in low-density cultures after 2 days in response to different conditioned media. Survival of thalamic cells was promoted to the same extent by all conditioned media including E19 thalamic conditioned medium ($p < 0.05$ when any treatment is compared with control, $p > 0.05$ when any treatment is compared with the others).

Abbreviations: as in Figure 4.4.


100
80
60
40
20
0
% Survival
*
*
*
*
control
E15THCM
E15CCM
E19THCM
E19CCM
Culture Medium

The thalamic viability promoted by conditioned media at low density was around the same level as the viability in high plating density. This suggested that at 2 DIV, cells survived at high plating density environment were due to the local release of diffusible trophic molecules. In addition, evidence that E19THCM was able to promote early thalamic cells at low density at the same level as other conditioned media suggested that its low-but-significant trophic effects on 5 DIV high-density thalamic cultures was not an artifact.

4. Testing the trophic factors productivity of the maturing thalamus

Intrinsic trophic efficacy of the thalamus is reduced as the tissue matures. In section 3.1, E19THCM promoted thalamic survival in a much lesser extent than E15THCM. Two possible explanations for this include:

1) A reduction in production of thalamic derived neurotrophic factors: the internal clock of the thalamus tells it not to produce the trophic factors required by it and start to depend on trophic factors from other sources.

2) A reduction or switch in the responsiveness to autocrine/paracrine thalamic derived trophic factors: thalamic cells may switch or narrow their trophic requirement and no longer respond to trophic factors produced by themselves.

However, my observations indicated that the second possibility is less likely because thalamic cells at 5 DIV (equivalent to P0 *in vivo*) were able to respond to the trophic factors that they produced at E15 (E15THCM) and still responded (although weakly) to conditioned medium from E19 thalamus. To test whether E19 thalamus is able to produce diffusible trophic factors or thalamic cells cultured for 5 DIV (around P0 *in vivo*) lose their ability to respond to the intrinsic factors, I derived two approaches to increase the amount of the released trophic factors from several neuronal tissues to be added to the dissociated thalamic cells: 1) by homogenisation technique and 2) by increasing activity of the explants that had been used for conditioning medium.

4.1 Effects of homogenated thalamic and cortical extracts on dissociated thalamic cells

In this experiment I used E15 and E19 thalamic or cortical extracts in an attempt to rescue dissociated thalamic cells that had been cultured for 5 DIV. However, none of these tissue extracts promoted thalamic survival of 5 DIV cells. Instead, some of them had toxic effects early on 2 DIV dissociated thalamic cells. In these 2 DIV cultures, viability of dissociated cells with the added E15 thalamic or E15 cortical extracts survived with a similar degree as control cultures (without added tissue extracts) (around 50%) while addition of E19 thalamic and E19 cortical extracts killed all dissociated cells.

To test whether the toxic effects of E19 thalamic and E19 cortical extracts is due to the high concentration of the extracts, I diluted E19 thalamic and E19 cortical extract in 1:10, 1:100, and 1:1000 of the first concentration I tested. None of these concentrations rescued thalamic dissociated cells.

4.2 Effects of thalamic and cortical conditioned medium of different ages; with K^+ added to the conditioned explants

When the first attempt to study the trophic effects of E19 thalamus by direct tissue extraction failed, I tried another approach to increase trophic production by increasing the activity and viability of E19 explants used for conditioning medium.

KCl has long been used for depolarising neuronal tissue and it hence increases neuronal activity. K^+ enhances production and release of trophic molecules from several types of neuronal tissue (Ghosh et al., 1994; Magowan and Price, 1996). More importantly, the addition of K^+ also promotes survival of certain neuronal populations (particularly thalamus) during particular developmental periods e.g. Magowan and Price (1996) showed that E19 thalamic explants cultured for 3 days did not survive but addition of K^+ rescued most of the thalamic cells in the explants.

As it is probable that the low trophic effects of E19THCM is due to the reduction in viability of the E19 thalamic explant used for conditioning medium, I thus added K^+ at concentration of 0.05mM, 0.5mM, 5mM, and 50mM either to medium used for conditioning explants of E15 thalamus, E19 thalamus, E15 cortex, E19 cortex, or directly to dissociated cultures. These are the concentrations used in Magowan and Price (1996). I then measured viability of both explants used for conditioning medium and dissociated thalamic cells in different conditioned media with added K^+.

4.2.1) Assessing the viability of explants that had been used for conditioning culture medium with the addition of K^+.

Before studying the viability of thalamic cells in conditioned media with added K^+, viability of explants cultured with different concentrations of K^+ was first assessed (Figure 4.6, Figure 4.7a). This was to test whether addition of K^+ modified explant viabilities and to see if the viabilities of dissociated cells with different conditioned media correlate with the viability of conditioned explants treated with K^+.

E19 and P2 thalamic explants without K^+ could not survive, and all cells in all explants with added 50mM K^+ died. Survival cortical explants treated with K^+ at concentration lower than 50mM were not significantly different from the viability E19 cortical explant without K^+ (viability = 70-80%). P2 cortical explants were also unaffected with 0.05-5mM K^+, but had a slight lower viability (60-75%). Survival of P2 thalamic explants was low and was not increased with K^+ treatment. Survival of E19 thalamic explants which is normally low in normal medium was increased with the addition of K^+ in a concentration-dependent manner.

4.2.2) Increased viability of thalamic cells in dissociation in E19THCM with added K^+

Except for the 50mM K^+ concentration which is the toxic dose which killed all cells, 2 DIV dissociated cells in culture medium with direct addition of K^+ in all other concentrations survive normally. Direct application of K^+ did not promote

Figure 4.6

Photomicrographs showing explants used for conditioning medium.

(a) E19 thalamic explant without addition of KCl: viability is poor and many cells become pyknotic.

(b) E19 thalamic explant with KCl: many cells still survive at 24h.

(c) E19 cortical explant: most cells have large nuclei and diffuse chromatin indicating that they survive in 24h culture.

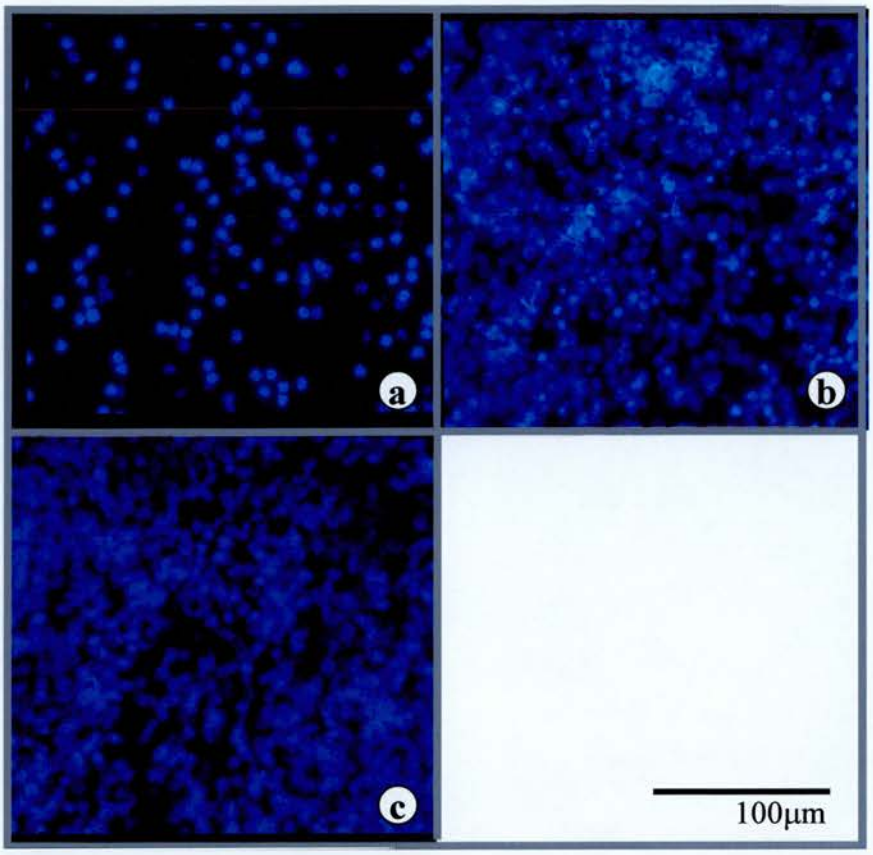
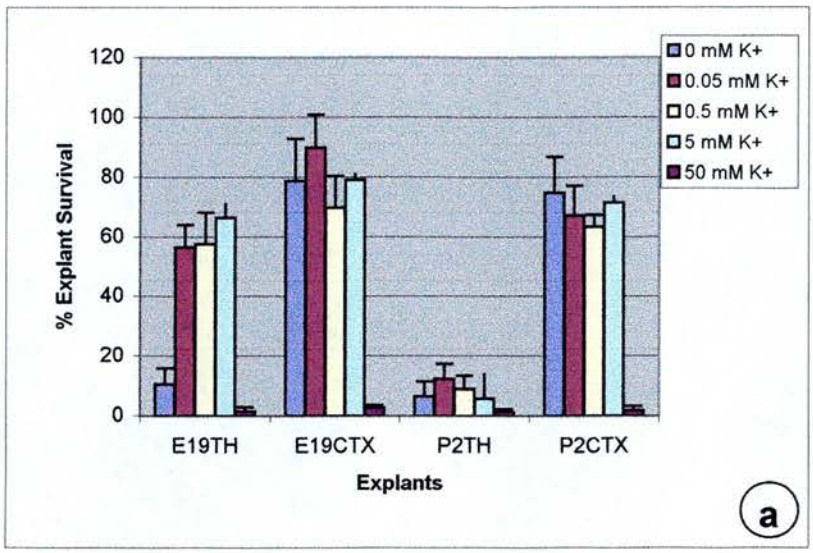

a
b
c
100μm

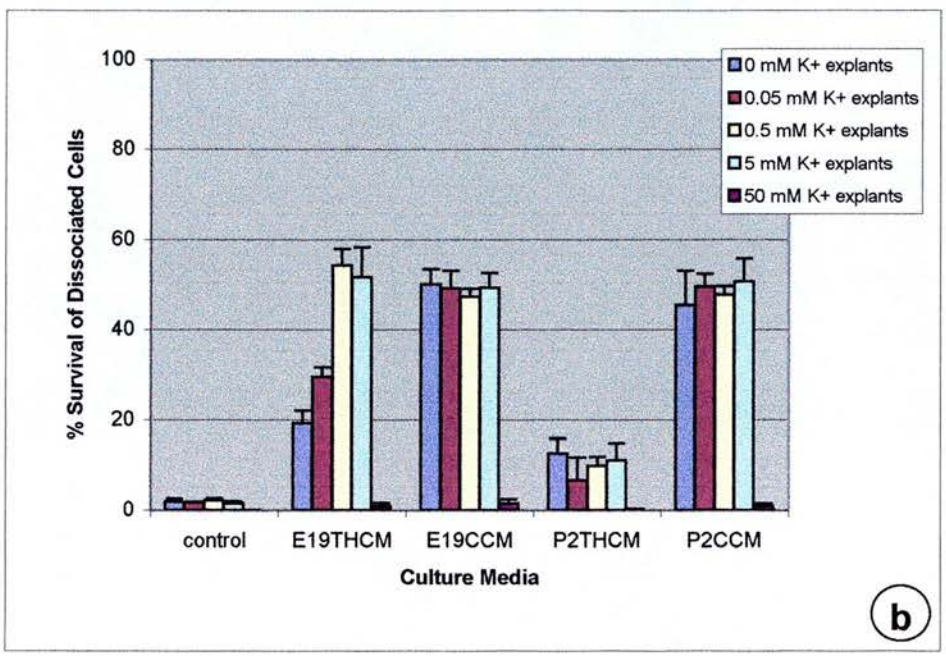
Figure 4.7

Graphs showing viability of (a) explants that had been used for conditioning medium with addition of K^+ and (b) dissociated thalamic cells after adding different conditioned media from tissues that had been pretreated with K^+. Addition of 50mM K^+ is toxic to both dissociated thalamic cells and cells in the explants.

(a) In E19 thalamic explants that have been used for conditioning medium, the survival was enhanced by the addition of K^+ in a dose-dependent manner after 24h in cultures. Addition of K^+ did not increase the survival P2 thalamic explants. Neither E19 nor P2 cortical explants respond to KCl.

(b) Dissociated thalamic cells in control medium do not respond to the addition of K^+ below 50mM.
Survival of dissociated thalamic cells cultured for 5 days was promoted by E19THCM-K^+ in a dose-dependent manner to the amount of K^+ that has been added in conditioning.
Addition of K^+ at the concentration below 50mM to the preconditioned medium did not change the trophic effects of P2THCM, E19CCM, or P2CCM.


120
100
80
60
40
20
0
% Explant Survival
0 mM K+
0.05 mM K+
0.5 mM K+
5 mM K+
50 mM K+
E19TH
E19CTX
P2TH
P2CTX
Explants
a


100
80
60
40
20
0
% Survival of Dissociated Cells
0 mM K+ explants
0.05 mM K+ explants
0.5 mM K+ explants
5 mM K+ explants
50 mM K+ explants
control
E19THCM
E19CCM
P2THCM
P2CCM
Culture Media
b

survival of 2 DIV thalamic cells. None survived at 5 DIV (see control group with added K^+ in Figure 4.7b). This study excluded the possibility that the increased viability of the dissociated cells in any media conditioned with added K^+ is due to the direct effects of K^+ but is due to the trophic release from the explants used for conditioning.

Thalamic and cortical explants at E19 and P2 were used to condition the media with different concentrations of added K^+. Conditioned media with K^+ were then added to 24h-dissociated thalamic cells. At 5 DIV, the survival of dissociated thalamic cells in E19THCM with added a K^+ (E19THCM-K) was increased ($p < 0.05$ control) (Figure 4.7b). This was dose-dependent (viabilities ranging from 0mM, 0.05mM, 0.5mM, 5mM K^+ in E19THCM-K = 19.24 $\pm$ 2.78 %, 29.48 $\pm$ 2.18 %, 54.32 $\pm$ 3.64 %, 51.7 $\pm$ 6.6 %). None of the media conditioned with 50mM K^+ promoted thalamic survival in dissociated cultures. E19 and P2 cortical conditioned media with added $\leq$ 0.5mM K^+ (E19CCM-K and P2CCM-K) promoted thalamic viability normally (around 50% survival). P2 thalamic conditioned medium showed a slight survival promoting effect on the dissociated cells (7-12 %) but P2-THCM-K^+ did not increase the dissociated cell survival.

5. Effects of thalamic and cortical conditioned medium on the population of GABA immunoreactive cells

The vast majority of cells in 2-day culture was completely negative for GABA (~ 83%). This suggested that the proportion of interneurons in cultures was low (similar to *in vivo*, Arcelli et al., 1997). In 5-day culture, most thalamic cells died while the surviving cells were mostly immunopositive to GABA antibody (Figure 4.8c, 4.9). It is less likely that the increase in GABAergic ratio in this culture was due to the *in vitro* differentiation inducing cells to become GABAergic, because 1) I rarely observed many dead cells that were immunopositive to GABA in this culture (see Figure 4.8c) and 2) the numbers of GABA positive cells in each window in this culture were always lower than the numbers of GABA positive cells in other cultures (comparing Figure 4.8c to Figure 4.8d). This implied that GABAergic neurons (interneurons) are less dependent on the external trophic support when compared

Figure 4.8

Photomicrograph showing immunohistochemistry for gamma-amino-butyric acid (GABA) in high-density dissociated thalamic culture.

(a) Negative control, when anti-GABA antibody is omitted.

(b) Negative control with pre-adsorbed GABA antibody; anti-GABA antibody was pre-incubated with GABA molecules (1:100) before reacting with the samples.

(c) Immunoreaction for GABA positive cells in 5-day thalamic cells cultured in normal culture medium (CM). Most cells die in normal culture medium and most surviving cells are GABA positive (as seen by dark brown color).

(d) Immunoreaction for GABA positive cells in 5-day thalamic cells cultured in cortical conditioned medium (CCM). Darkly labeled cells, lightly labeled, and negatively labeled cells are shown by red, green and white arrows accordingly. Darkly labeled cells are present in a smaller proportion of the population than in normal medium.

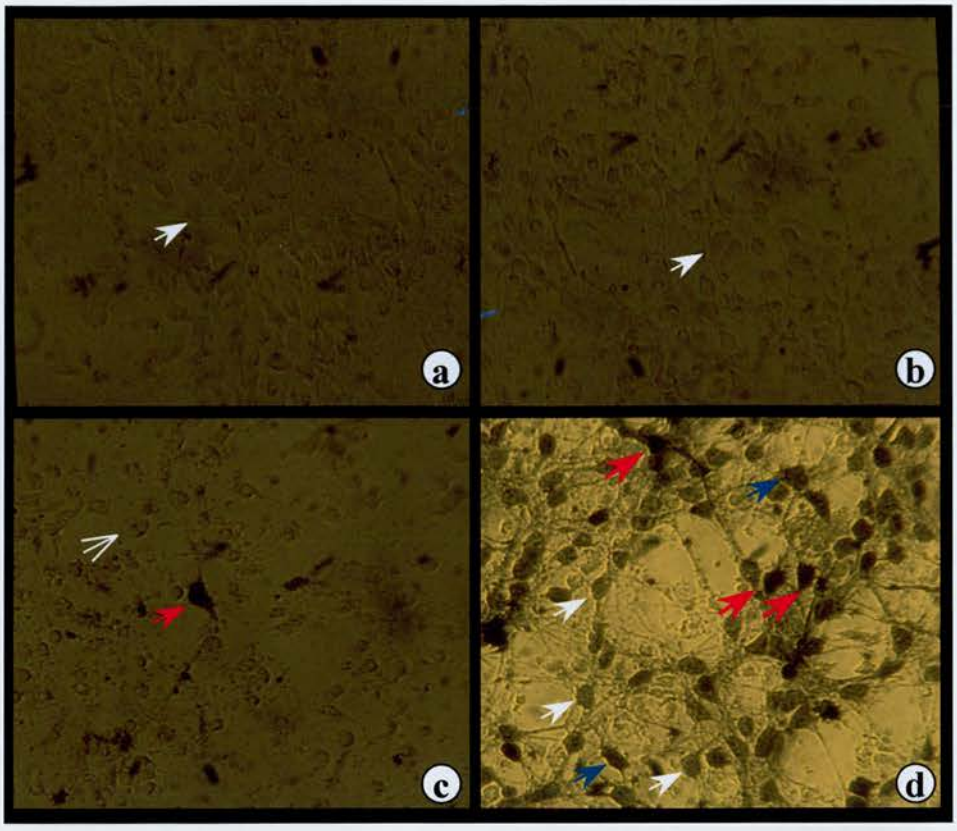
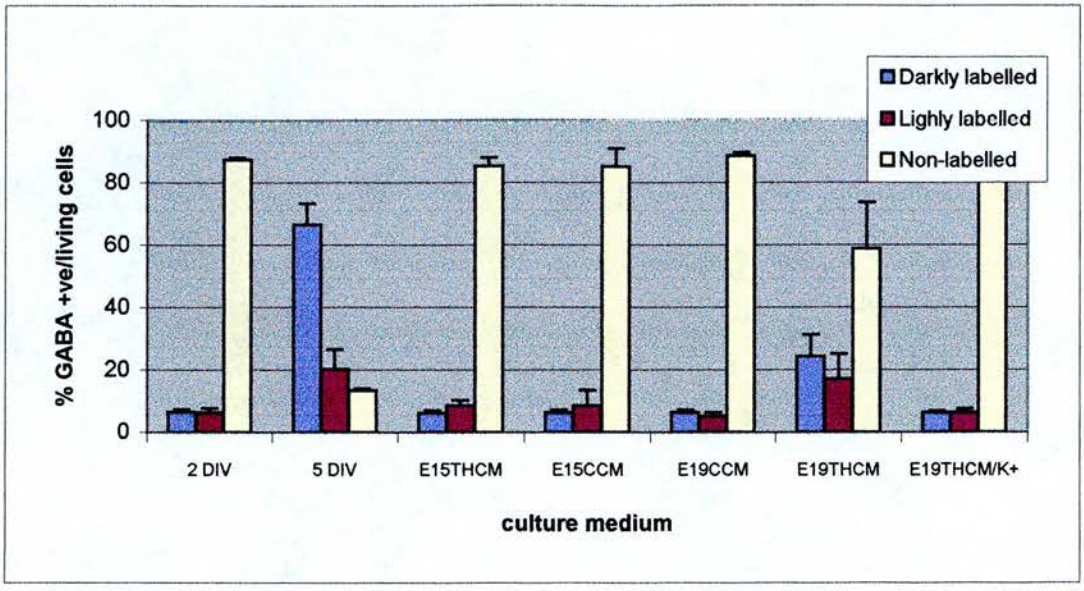

a
b
c
d
100μm

Figure 4.9

Graphs showing distribution of GABA positive cells in thalamic cultures. When comparing percentages of GABA positive cells in proportion to total living cells in culture, GABA positive cells (supposedly interneurons) are present in small proportion in 2-day with normal medium cultures and in 5-day cultures with E15THCM, E15CCM, E19CCM, and E19THCM-K^+ (around up to 10% darkly labeled ratio or lightly labeled cells), while in 5-day cultures with normal or with E19THCM (without K^+), proportions of GABA positive cells are increase as the non-GABAergic cells die without enough trophic support.


Darkly labelled
Lighly labelled
Non-labelled
% GABA +ve/living cells
100
80
60
40
20
0
2 DIV
5 DIV
E15THCM
E15CCM
E19CCM
E19THCM
E19THCM/K+
culture medium

with other type of cells. Application of E15THCM (but not E19THCM), E15 or E19CCM promoted overall thalamic survival and the proportions of GABAergic cells (both darkly and lightly labeled populations) were in the same proportions with surviving cells as in 2-day culture (without statistical differences) (Figure 4.9). These cultures had lower percentages of GABAergic cells than 5-day cultures without conditioned media. However, in 5-day cultures with E19THCM, where overall cell survival was promoted slightly (< 10%), the ratio of living GABAergic positive cells to the total of living cells was lower than in a 5-day control but significantly higher than with other conditioned media. This was possibly because the E19THCM had low trophic effect on promotion of non-GABAergic cell survival and the result was less likely to be due to any prominent E19THCM promotion of GABAergic populations.

Discussion

Thalamic cells that have been in culture for longer than 3 days cannot survive without external trophic support. This trophic requirement lasts until at least around the time of birth (5-6 DIV). Removal of the diffusible trophic factors at any time during the culture period would result in an increase in cell death. These trophic factors, although currently unknown, likely include molecules that are sensitive to heat inactivation such as proteins. The trophic support, however, is not only present in the major target (cerebral cortex), but it can also be found in conditioned media obtained from other main regions. The sources of these trophic molecules are restricted to neuronal tissues and the molecules are not found in other non-neuronal tissue such as heart or liver. Whether or not the trophic effects found within different neuronal sources come from the same neurotrophic factors is still unclear. It is also not known whether the mechanisms that promote thalamic survival are likely to control neuronal outgrowth or vice versa. Additionally, it is important to discuss here the role of the intrinsic property of the thalamus before and after the target innervation period and the switch in its requirements.

Possible trophic substances to be presented in conditioned medium

Many types of conditioned media applied to E15 low-density thalamic cells (where there are less cell-cell interactions) can mimic the trophic effect presented in E15 high plating density cultures. The conditioned media must therefore contain one or more essential trophic substance(s). Withdrawal of these substances produces similar results to the withdrawal of neurotrophins or FGF deprivation that has been previously demonstrated (Lotto et al., 1997). Together with the *in vitro* BDNF experiments done in collaboration with Dr. Beau Lotto (see Chapter 3), this result strongly ensured that at least one factor that thalamic cells require from the conditioned medium is likely to be a neurotrophin (i.e. BDNF in particular), and I further tested whether the trophic molecule is likely to be protein by heat treatment. I found that viability of thalamic cells was reduced if the cortical conditioned medium is heated for 30 minutes. However, there is still a possibility that other trophic

factors, in addition to neurotrophins, are involved. This is because heat inactivation cannot completely abolish the neurotrophic effects of the conditioned medium, and the molecules seem to be relatively stable as short exposure (3 minutes) of heat cannot reduce their trophic effects.

Some extracellular matrix substances are among candidates for these trophic substances. Several members of proteoglycans such as chondroitin sulfates, and heparan sulfates and kerantan sulfates abundantly found in neuronal tissues may have either growth inhibiting or growth promoting effects, depending on the types of the molecules and the populations of cells (i.e. one type of molecules may have different effects when present in different populations) (Emerling and Lander, 1996). Generally, most proteoglycans are found to inhibit neurite outgrowth in many neuronal systems (see review by Holm and Isacson 1999) but heparan sulfate was found to have growth promoting effects on thalamic axons (Kinnunen et al., 1999). Additionally, this molecule also helps increased bioactivity of basic-fibroblast growth factor (bFGF) (Nissen et al., 1999), which is also known to promote thalamic survival and outgrowth (Lotto et al., 1997; Lotto and Price, 1995). More importantly, most chondroitin sulfates are relatively more stable to temperature treatment than ordinary proteins or peptides (Volpi et al., 1999). Moreover, Lander and colleagues showed that cortical conditioned medium contains chondroitin sulfate proteoglycan (Lander et al., 1998). This might help to explain the remaining trophic effects of the cortical conditioned medium after heat treatment.

Specificity of trophic factors in conditioned medium from other tissues

Hisanaga and Sharp (1990) first showed that in addition to target cortical neurons, non-target cerebellar cells also produce a prominent trophic effect on thalamic neurons. In this study, I investigated further whether other structures also have a stimulatory effect on thalamic survival. I found the trophic factors required by thalamic cells are likely to be present only in neuronal tissues. Thus, 1) cells responsible for producing such trophic factors are specifically or preferentially either neurons or glia and 2) the level of factors produced may be different in different CNS

structures. Two candidates that fit both characteristics are neurotransmitters and neurotrophins.

Although several CNS tissues tested produced trophic effects on maturing thalamic cells, it is not know whether these are the same trophic factors that specifically promote thalamic survival. However, the choices of possible trophic molecules essential for survival of the late thalamus may be limited since thalamic neurons lose trophic promiscuity and have more specific trophic requirement as they mature. For example, the cells lost their ability to respond to several neurotrophins but still require BDNF as they mature (Chapter 2, Lotto et al., 1997). This suggests that trophic molecules that are found in different CNS tissues, which promote thalamic survival, could be the same molecules. However, to precisely answer this question, isolation and identification these trophic molecules individually presented in each CNS structure are required.

In vitro, thalamic cells respond to various sources of trophic factors, however, this does not necessary mean that the cortical target is not the major/specific source of trophic supply for thalamic cells in *in vivo*. Apart from autocrine responses, it is believed that neurons obtain their trophic support via their target innervation (Oppenheim, 1999). As the animal matures, production of trophic factors for thalamic cells need thalamocortical activity (Ghosh, 1996). In addition, trophic factors produced by the thalamus itself are reduced as the thalamus matures (comparing the trophic effects of E15THCM and E19THCM), and this requirement seems to correspond with the period of target innervation and programmed cell death. Therefore, it is still acceptable to believe that the majority of the late embryonic thalamic cells require and obtain trophic support from the cortical target.

Study of trophic effects of K^+ and homogenated thalamic and cortical extracts

K^+ increases the viability of the thalamic explants but not the viability of thalamic cells in dissociation. A possible explanation is that the action of K^+ needs an intact and high degree of thalamic organisation (e.g. intrathalamic connections, and axonal formation) which is preserved in the explants but not in dissociation.

Dissociated thalamic cells have to adapt in to a new environment and may have lost their original intrinsic activity. Thalamic cells taken from E15 embryos mostly die after 3 days in dissociated cultures while most still survive in explant cultures.

Tissue extracts have been introduced in several studies for the isolation and identification of neurotrophic factors (Yin et al., 1992; Sato et al., 1997). For example, survival of chick motoneurons is promoted by chick muscle extracts (see Sato et al., 1997) and conversely, extracts of nerve tissue can enhance the development of chick embryonic muscle (Oh, 1976). Although many have found trophic effects in many types of tissue extracts, the identification of the trophic molecules is often unsuccessful. Contrary to those reports, I did not find any trophic effects of neuronal tissue extracts on thalamic survival. In addition, E19 cortical extracts were even toxic to the thalamic cells. Survival of thalamic neurons might depend on the balance between growth promoting and growth inhibiting factors. In this case, the effects of neurotoxic substances may overcome the effects of trophic factors.

Regulation of thalamic survival by activity-dependent mechanisms and trophic factors signaling: A possible interaction of the two.

The interaction between activity and neurotrophins is essential for neuronal growth and survival (McAllister et al., 1996). It is not known whether interaction between both are in linear, ordered relationship (activity promotes production/secretion of trophic factors; activity → trophic factors → survival, or neurons may primarily need activity to survive but trophic factors may help strengthen the synapse formation and increase the survival; trophic factors → activity → survival). It is also possible that neuronal survival may need both trophic factors and maintenance of activity in a complex mechanism (neurotrophic factors + activity → survival) (see McAllister et al., 1996). Increasing evidence suggests that activity enhances trophic release and hence promotes survival. Depolarising agents such as KCl and glutamate have been shown to enhance survival of central neurons *in vitro* (Ghosh et al., 1994; Cohen-Cory et al., 1991; Meyer-Franke et al., 1995). This mechanism has been shown to involve intracellular Ca^{++} signaling and trophic

factors release (Finkbeiner and Greenberg, 1996; Ghosh et al., 1994; Cohen-Cory et al., 1991; Meyer-Franke et al., 1995; Magowan and Price, 1996). Both *in vitro and in vivo*, neurotrophin production and secretion are regulated by electrical and synaptic activity (Castren et al., 1992; Lindholm et al., 1994; Schoups et al., 1995; Blochl and Thoenen, 1995; 1996; Goodman et al., 1996).

However, depolarisations by KCl and by glutamate act via different Ca^{++} channels. Trophic effects of K^+ is thought to act via voltage-sensitive calcium channels (VSCCs) and induce release of neurotrophic factors (Ghosh et al., 1994). In my experiment, I have also shown that K^+ depolarisation can induce release of trophic substance(s) in the E19 embryonic thalamic explants and hence promote survival of dissociated thalamic neurons. In contrast, the mechanism of trophic effects of glutamate *in vivo* is still unclear. Ghosh et al. (1994) found a small neurotrophic effect by activation of kainate receptors but not by NMDA receptors. In contrast, by experiment applying APV antagonist for NMDA receptor, Magowan and Price found that glutamate likely to promote thalamic survival via NMDA receptor (unpublished results). Whether or not glutamate increases trophic factors to promote neuronal survival remain to be investigated.

In vivo, survival of thalamic neurons may require the release of neurotransmitter such as glutamate (from the thalamic neurons themselves, from their afferent inputs, or from the retrograde target-dependent activity) or electrical depolarisation, which consequently increase the release of trophic molecules. However, neither K^+, glutamate, nor neurotrophins are sufficient to prolong the trophic effects on thalamic neurons at the postnatal stage (Magowan and Price, 1996; Magowan and Price, unpublised data; Lotto et al., 1997). The regulation of programmed cell death may need additional control, the intrinsic regulation of timing such as the control of bcl-2 expression (see below).

Growing thalamus: Thalamic viability, age related trophic requirement and possible role of extracellular matrix

The progressive loss of the ability of dissociated thalamic cells to survive with time in culture may be due to several reasons. The first simplest explanation is that cells are dying over the time in culture as a result of the consumption of nutrients and accumulation of waste/toxic substance in culture medium. However, this is unlikely to be the case since replacing culture medium cannot rescue thalamic cells that have been cultured for longer than 3 days and the thalamic cells are strongly dependent on the conditioned medium.

The peak of cell death of thalamic cells in culture (E17-E18 *in vitro*) preceeds the normal period of thalamic PCD (from P1) *in vivo*. This can be explained by 1) the adaptation to the new environment that may induce the maturation or susceptibility to death of thalamic cells, 2) the lack of trophic substances which are normally found in the thalamus at E17-P0. *In vivo*, it is possible that this late gestation may be the window period that thalamic cells prepare themselves to become dependent on their cortical target. Concomitantly, brain is increasing extracellular space and producing extracellular matrix, which includes proteoglycans (Holm and Isacson, 1999). In many cases, extracellular matrix is thought to be neuronal inhibitive to neurite outgrowth although it is still controversial (Holm and Isacson, 1999; Nissen et al., 1999). It is possible that the increase in extracellular matrix may reduce cell-cell interaction and neuronal activity within the thalamus and reduces the intrinsic trophic production, and thus forces the thalamic cells to become dependent on their cortical target via thalamocortical axons.

Switching in trophic dependence of thalamic cells: Specificity in cortical target dependency but not specificity in trophic responsiveness

At the time corresponding to *in vivo* E17-P0, cultured thalamic cells progressively lose their ability to survive and grow *in vitro*. Many have previously described cortex-derived growth factors may be the major influence to the regulation of thalamic survival and growth which corresponding to time of cortical target

innervation (Cunningham et al., 1987; Price et al., 1995). Similar findings on regulation of neuronal survival by target were also reported in other neuronal systems. Many types of neurons are transiently supported by other neurotrophic factors early in their development before they switch their trophic responsiveness and become dependent to their target derived factors (Davies, 1994a). However, I found that thalamic cells aged at the time of target innervation did not switch off the ability to response to the self-produced (thalamic-derived) neurotrophic factors. This suggests that target dependency does not always correspond with selective responsiveness to the trophic factors released from the target tissue. This finding does not correspond to the previously described trophic switch hypothesis which states that cells would lose both responsiveness and self production of theirs own trophic factors before becoming dependent on target (Davies, 1994a).

Cortical conditioned media of different ages (E15-P2) promote thalamic survival to a similar degree. However, the trophic effects of the condition medium from the thalamus of different ages are varied. Thalamic cells responded to E19THCM but in a much lower degree than the E15THCM. With the addition of K^+ on the E19 thalamic explants, E19THCM-K had similar effects as E15THCM. This suggests that thalamic cells did not lose ability to respond to the trophic factors released endogenously but instead, they may lose the ability to produce sufficient quantities of trophic supply. Intrinsic factors may set the clock for thalamus when they have to become dependent on the target by the regulation of trophic production. This regulation for the loss in the intrinsic property in the maturing thalamus will be discussed below.

Intrinsic mechanism regulating thalamic survival and signaling for switching in trophic dependence

Intrinsic mechanisms may play a key role in regulation of thalamic viability before their axons innervate their target (Davies, 1994a). The intrinsic signaling in addition to the extrinsic factor signaling (Holm and Isacson, 1999) may trigger the loss in the intrinsic viability. This intrinsic regulation involves the local cellular activities such as metabolism, electrical or biochemical activities within the cells or

communication, and genetic internal clock (Magowan and Price, 1996; Holm and Isacson, 1999). Certain apoptotic regulating genes may be involved in this thalamic survival regulation. For example, *bcl-2* (anti-apoptotic) and possibly *bax* (pro-apoptotic) genes are thought to be in a central mechanism regulating PCD in many neuronal systems (Pettman and Henderson 1998). *bcl-2* is expressed in the CNS during the middle to late gestational period and its expression is down regulated at the time of birth, persisting in regions with late differentiation, such as the dentate gyrus (Merry et al., 1994). This correlates with the period of *in vivo* peak of the thalamic PCD (see Chapter 3). Bcl-2 production declines with age in most CNS neurons (Holm and Isacson, 1999). The pattern of the *bax/bcl-2* expression ratio corresponds with the timing of neuronal death in the rat cortex and thalamus (Mooney and Miller, 1999). Furthermore, Bcl-2 has a neuroprotective effect against neurotrophin deprivation in many types of neurons and some think that it is the intrinsic instructive gene controlling the time of PCD (Allsopp et al., 1993; Holm and Isacson, 1999).

Outgrowth vs. survival promoting effects of trophic factors in conditioned medium

Trophic derived growth factors obtained from non-target E19CBCM, E15THCM, and K^+-induced E19THCM (E19THCM-K) increased thalamic cell survival at the same degree as the target E19CCM. However, previous report showed that thalamus co-cultured with different CNS tissues had different degrees of thalamic outgrowth. Cerebellum and thalamic explants enhanced less outgrowth than cerebral cortex explants did (Lotto and Price, 1996). Despite the fact that less viable cells produce less outgrowth (Magowan and Price, 1997), the converse is not necessarily true: enhanced viability does not necessarily produce enhanced outgrowth. The relationship between thalamic viability and growth may not be simple. For example, K^+ promotes both survival and outgrowth of E13-E17 thalamic explants but only promotes survival without enhancing neurite outgrowth of thalamic explants at E19. It is thus possible that outgrowth and survival promoting effects of K^+ are mediated by different proteins (Graham & Burgoyne, 1993; Magowan and Price, 1997).

It is not known whether these trophic factors rescuing thalamic cells are the same as that found in all conditioned mediums from several neuronal sources used in this study. This issue remains to be further investigated.

Conditioned medium did not affect GABAergic subpopulation in the thalamus

The thalamus consists of several subpopulations of neurons. Most thalamic neurons are projecting cells that innervate cortex (thalamocortical cells) while some smaller proportions of thalamic cells are GABAergic interneurons. My experiments aimed at 1) testing whether the thalamocortical projecting neurons obtain neurotrophic support from their cortical target, in particular, the target at the age of thalamocortical innervation (E19CCM) and 2) testing whether GABAergic thalamic interneurons which axons are confined to thalamus were independent on cortical-derived trophic factors but possibly on thalamic derived trophic factors. I first predicted that 1) E19CCM would increase more proportion of projecting neurons than other test conditioned media and 2) E15THCM, E19THCM, E19THCM-K would either promote or sustain the same level of GABAergic neurons.

Thalamic cells without conditioned medium die at 5DIV, 2DIV (E17 *in vitro*) were thus used as control samples. This may cause an inaccuracy in the estimation of the proportion of GABAergic cells because the E17 *in vitro* cells may be less differentiated than E19 *in vitro* cells. However, neither E15CCM nor E19CCM altered the proportions of GABAergic and non-GABAergic populations in 5 DIV cultures. Percentages of GABAergic populations were also similar among E15THCM, and CCMs. This suggests that all conditioned mediums may only sustain overall viability of thalamic cells, does not selectively rescue specific subpopulations or involve in GABAergic differentiation.

Considering the GABAergic population, the surviving thalamic cells at 5 DIV are mostly GABAergic cells. Numbers of GABAergic cells are relatively and similarly low throughout all treatments. This suggests that the GABAergic cells may be independent to any conditioned medium.

Summary

In conclusion, I found that trophic dependency in the thalamus is not only regulated by extrinsic factors but also by the intrinsic factors. The switch in trophic dependence to the target support may still exist but with different mechanisms i.e. thalamic cells may not reduce the trophic responsiveness but the production of sufficient intrinsic trophic factors. Thalamic cells also respond to trophic factors present in other CNS tissues than the thalamus and the cortex.

There are other important questions remain to be further investigated. These include: the identification of the intrinsic factors regulating thalamic survival, the mechanisms underlying switching of thalamic cells to extrinsic dependence, and the characterisation of the neurotrophic molecules in condition media obtained from cerebral cortex as well as different neuronal sources of tissue.

Chapter 5: *In vitro* studies in the thalamus of mice lacking neurotrophin tyrosine kinase receptors

Introduction

Previous studies have shown that neurotrophin signaling is required for survival of late embryonic and postnatal thalamic neurons that have innervated the cortex (see Chapter 3; Alcantara et al., 1997). In mice lacking Trk receptor genes, such as *trkB-/-* mice, there is a significant increase in cell death in different regions of the thalamus around E17-P1 (see Chapter 3) and in late postnatal life (after P8, Alcantara et al., 1997). However, the increases in these *in vivo* studies are subtle (occurring at low rates), and no marked increase in cell death appeared before E15. Moreover, the gross morphology of the thalamus of these mutants still appeared normal (Klein et al., 1989). Possible explanations for the subtlety of the effects include compensation by other neurotrophins or an overlap in the functions of Trk receptors (Ernfors et al., 1992; Merlio et al., 1992).

In a system isolated in tissue culture, any compensation mechanisms involving neighboring or target tissue would be reduced. Organotypic slice cultures have previously been used to study the effects of axotomy on the survival of developing *trkB-/-* hippocampal neurons that have already reached their target fields (Alcantara et al., 1997). In thalamus, the *in vitro* defects resulting from Trk knockouts have not been studied. In this Chapter, I used an *in vitro* approach to investigate the survival of thalamic neurons of mice with mutated *trkB* or *trkC* genes at the ages before and after innervation of the cortex. Ideally, I would have carried out a dissociation technique that would also reduce the chance of compensatory effects produced intrinsically within the thalamus. However, there are difficulties since I would have had to dissociate each embryonic thalamus individually and genotype it before cells were plated and labeled. This approach seemed impossible. Instead, organotypic thalamic explants were used in this study and I identified their genotypes retrospectively by PCR.

In the first set of experiments, I isolated E15 thalamus of wild type, *trkB-/-*, and *trkC-/-* embryos to compare their viability *in vitro*. The thalamus at this age has begun to extend its fibers but they have not reached the cortical plate (Rennie et al., 1994; Auladell et al., 1999). This experiment was to investigate if the thalamus of the mutant mice depends on Trk signaling *in vitro* at this early stage in the absence of neighboring or target tissues as compared to *in vivo* where there are no differences in rates of cell death among the wild types and the mutants. Next, I cultured E19 mutant and wild type thalamic explants to observe their survival at the period of innervation. However, the survival of the thalamic explants of wild type, *trkB-/-*, and *trkC-/-* embryos are low at this later age and this made it difficult to observe any differences in the viability in mutants (see Results).

As a result of the low survival rates and difficulties in comparing the survival of the older wild type and mutant thalamic explants, I added K^+ to the E19 explants to increase their survival to the level at which they can be compared. K^+ is widely used to non-specifically depolarise neuronal membranes and enhance levels of neural activity, which will be increasing in this perinatal period in the thalamus, largely due to the onset of spontaneous activity in the peripheral sensory receptors (Shatz and Stryker, 1988). Activity can then stimulate the embryonic thalamus to grow and survive (Magowan and Price, 1996). However, the mechanisms by which K^+ promotes thalamic survival may be complex and the K^+ addition may trigger production of several other trophic signaling molecules than neurotrophins (Ghosh et al., 1994; Zafra et al., 1992). The result of this experiment thus requires careful interpretation.

Materials and Methods

Animal preparation

Mice heterozygous for targeted mutations in *trkB* or in *trkC* sequences encoding the catalytic domains of the TrkB or TrkC tyrosine kinase receptor isoforms (Klein et al., 1993; 1994) were used. Heterozygous *trkB* or *trkC* mice were mated to give wild type, heterozygous, and homozygous embryos. Embryos were removed at the assigned stage of pregnancy. Parts of the embryos (either legs or tails) were removed for PCR genotyping (see Chapter 3). Wild type and homozygous (*trkB-/-* or *trkC-/-*) thalami were dissected from foetal brains of E15 and E19 mice.

Explant culture procedure & histology

The thalamic explants were sliced on a McIlwain tissue chopper at 350 microns and placed in pre-incubated defined serum-free culture medium (Romijn et al., 1984). After culturing for 3 days at 37°C in 5% CO_2 with 95% humidity, the tissues were fixed in 4% paraformaldehyde and embedded. Embedding processes were done as described in Chapter 2. Sections were cut at 10μm and mounted on poly-$_L$-lysine coated slides.

Addition of K^+

5mM KCl (the concentration known to promote thalamic survival, Magowan and Price, 1996) was added to the serum-free culture medium used for wild type and mutant E19 thalamic explants.

Counts and analysis

To assess the viability of organotypic thalamic explants that had been cultured alone for three days, four explants of each age tested (E15 and E19) were fixed, the slides of the explants were stained with 1μg/ml Hoechst, and mounted with Vectashield (Vector). For each of five equally spaced sections taken from

approximately the middle of each explant, the number of pyknotic cells (dead or dying cells) per total number of cells seen in the 10 x 10 μm square grid window were counted. Viabilities of the wild type thalamic explants either versus the *trkB-/-* explants or versus the *trkC-/-* explants were compared using Students' t-test.

Results

1. E15 thalamic explants

Similar to the previous findings, cells of the E15 wild type thalamic explants cultured for 3 days maintained high levels of viability (80.62 $\pm$ 5.2%, n = 4). At this age, survival in the explants of mutant thalami (both *trkB-/-* and *trkC-/-*) was about the same level as that in wild type explants (Figure 5.1, n = 4 each, viability = 83.37 $\pm$ 3.12 % and 82.66 $\pm$ 3.5 % respectively). There was no significant difference in survival among these explants ($p > 0.05$). These results suggested that TrkB and TrkC signaling are not required for survival of thalamic cells of this age, which is before target innervation and target dependence.

2. E19 thalamic explants, without K^+

At E19, the age around the time of cortical innervation, survival of thalamic cells in wild type and in mutant explant cultures was poor (Figure 5.2a, 5.3a; 9.73 $\pm$ 4.2% for wild type, 7.06 $\pm$ 1.93 % for *trkB-/-*, and 7.5 $\pm$ 1.22 % for *trkC-/-*; n = 4 each).

There was no statistical difference in survival among these thalami. However, since the percentages of surviving cells were low, those surviving may represent only certain subpopulations which do not require these neurotrophic signaling in vivo. Thus it was not known if the rest of the cells at E19 that can normally be rescued by K^+ require TrkB or TrkC signaling. I then used K^+ at 5mM (the KCl concentration known to produce the highest thalamic cell viability) to increase thalamic activity to compare the viability in wild types and mutants.

3. E19 thalamic explants, with K^+

With the addition of K^+ to serum-free culture medium to promote survival of the mutant and wild type thalami, I predicted that 1) survival of all thalamic cells

Figure 5.1

Graph showing viability of E15 wild type and the *trk* mutant thalamic explants: survival of thalamic explants from both trkB-/- and trkC-/- embryos is as high as that of the wild type. No significant difference is found.

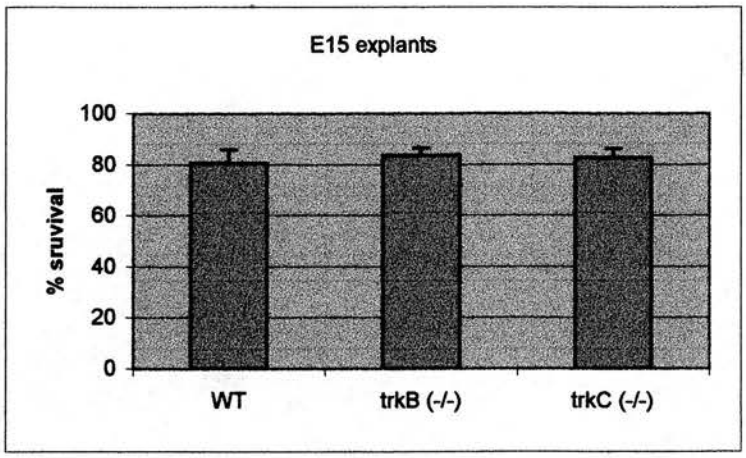

E15 explants
% sruvival
100
80
60
40
20
0
WT
trkB (-/-)
trkC (-/-)

Figure 5.2

Photomicrographs of thalamic explants stained with Hoechst.

(a) E19 wild type explant without K^+ treatment; most cells die. (b) E19 wild type explant with 5mM K^+. (c) E19 *trkB-/-* thalamic explant with 5mM K^+. (d) E19 *trkC-/-* thalamic explant with 5mM K^+. Over 60-70% of cells in (b)-(d) survive. Red arrowheads = pyknotic cells, while green arrowheads = viable cells.

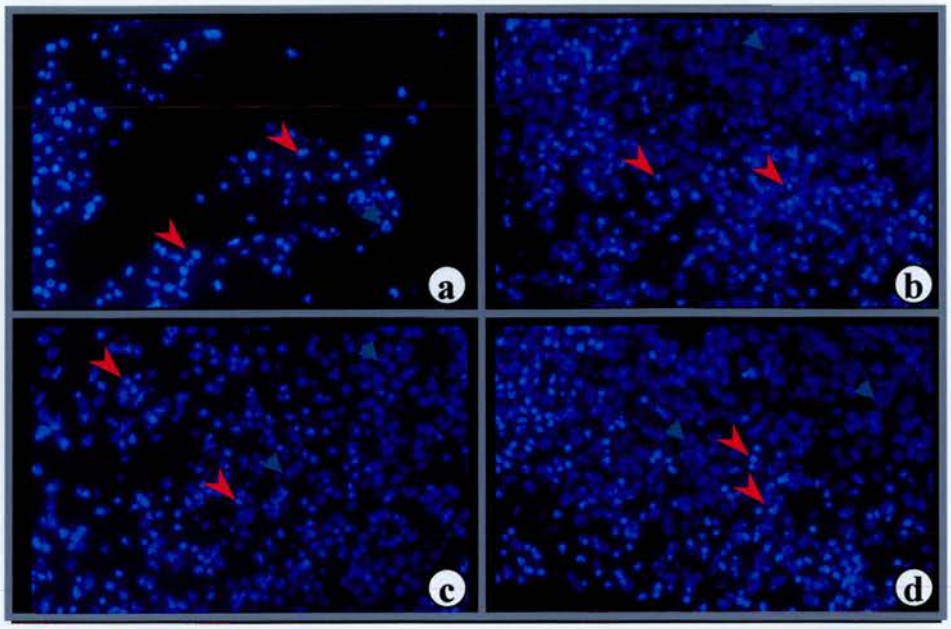
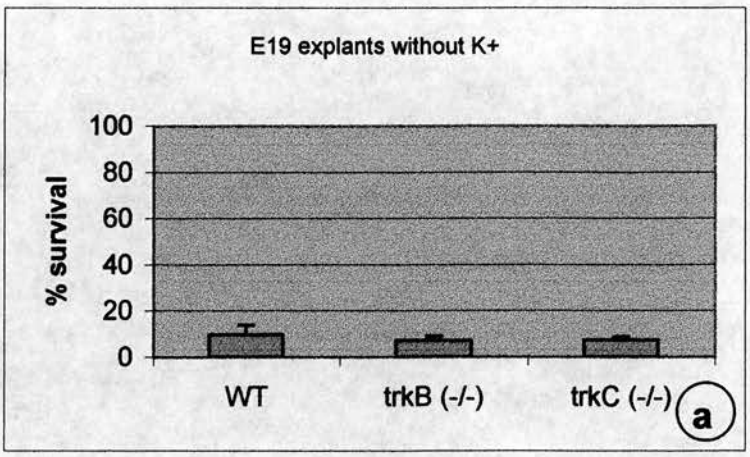
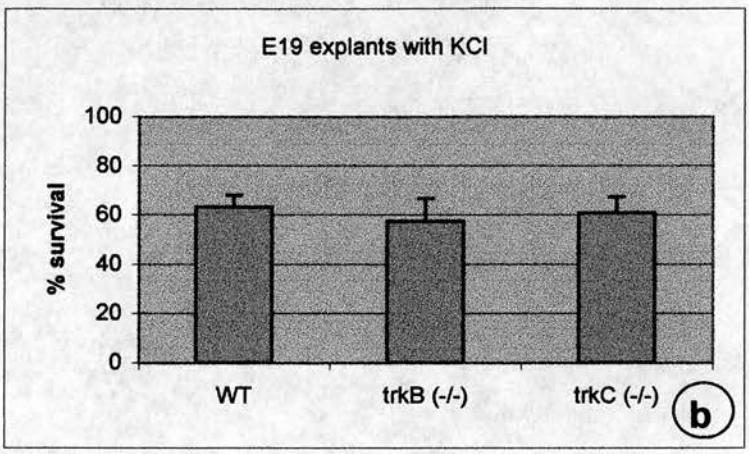

a
b
c
d
100μm

Figure 5.3

Graphs showing viability of E19 wild type and the *trk* mutant thalamic explants.
(a) E19 thalamic explants without K^+ treatment: most thalamic cells in either wild type or the mutants die. No significant difference in survival rate is found.
(b) E19 thalamic explants with K^+ treatment: K^+ promotes thalamic viability in both wild type and mutant explants equally. No significant difference is found.


E19 explants without K+
% survival
100
80
60
40
20
0
WT
trkB (-/-)
trkC (-/-)
a


E19 explants with KCl
% survival
100
80
60
40
20
0
WT
trkB (-/-)
trkC (-/-)
b

should be increased due to the increased activity; 2) if this K^+ effect is modulated by the release of neurotrophins (such as BDNF, NT-4/5, or NT-3), the thalamic explants from *trkB-/-* and *trkC-/-* mice should show less survival than those of wild type mice.

In this experiment, I found that the viability of thalamic cells in E19 explants was markedly promoted in wild type, *trkB-/-*, and *trkC-/-* explants (Figure 5.2b-d, 5.2b, n = 4 each; 63.26 + 4.85 %, 57.46 + 9.25 %, and 60.92 + 6.42% respectively). This indicates that the survival of thalamic cells at this age is activity dependent and that this activity dependent survival is not mediated by either TrkB or TrkC. Moreover, I failed to show the increase in cell death of *trkB-/-* thalamus which I previously demonstrated *in vivo*. No statistical differences in survival among the E19 thalamic explants of wild type, *trkB-/-*, and *trkC-/-* mice in the presence of K^+ were observed. Some possible explanations will be discussed below.

Discussion

The main purpose of this study was to assess the viability of embryonic thalamic cells from mice lacking TrkB or TrkC *in vitro*. Initially, I cultured the explants with the intention of establishing an *in vitro* system for further studies of the mutant tissue. Organotypic culture would normally provide an isolated system for specific studies such as the effects of K^+ depolarisation (Magowan and Price, 1996), the thalamocortical interaction (Lotto and Price, 1996) and target removal (Alcantara et al., 1997), the release and survival response to trophic factors (Lotto and Price, 1996), and the addition of neurotrophins and blocking antibodies (Ghosh et al., 1994; Segal et al., 1992). However, I found that there are several difficulties with carrying out such studies on the knockout tissues. For example, 1) it is a tedious process without knowing the genotype before putting tissue in to cultures, 2) the number of samples of particularly the homozygous tissues obtained in each experiment are variable and often low, 3) these variable number of samples of unknown genotype make it difficult to perform experiments with many variables/conditions, such as varying doses, and 4) the amounts of a test substance needed are high since the volumes for explant cultures are large (2.5ml culture medium per 2-4 explants, compared with 100µl per well in dissociated cell culture).

Thus, in this chapter, I did only simple pilot studies and analysis of the TrkB and TrkC knockout thalami in culture as follows: 1) I analysed thalamic viability in mutants taken before and after the period of cortical innervation in isolation and 2) I studied the effects of K^+ depolarisation (using the concentration which is known to promote thalamic survival, Magowan and Price, 1996) to test whether this required Trk signaling to promote cell survival.

1. Time and factors that control survival of thalamic explant

In wild type control experiments, the viability of the young E15 thalamic explants was high, corresponding to the low cell death rate *in vivo* (see Chapter 3) and high viability of E15 dissociated cells in culture (see Chapter 3 and 4; Lotto et

al., 1997). Together, this suggests that the factors sufficient to support the viability of the early embryonic thalamus are produced within the thalamus itself. *In vivo*, the target may have little or no influence on thalamic viability at this early embryonic age of E15 (Price et al., 1995). In addition, confirming the previous report (Magowan and Price, 1996), thalamic viability decreased in older explants at around the time of cortical innervation and in the perinatal period.

Increasing evidence shows that thalamic neurons become more demanding as the thalamus matures. Most of the older (> E19) thalamic cells in dissociated culture (Chapter 4) and in explants die as they age (see Figure 5.2b). It has been proposed that survival of thalamic cells at this age is regulated by both intrinsic and target dependent mechanisms (Lotto et al., 1997) and exhibit many interesting characteristics: i.e.1) the survival of the cells is highly sensitive to and transiently regulated by activity-dependent mechanisms (Magowan and Price, 1996). 2) E15 thalamic cells in culture up until E19-P1 (4-6 DIV) require BDNF signaling and many neuronal tissue-derived trophic factors (including cortical- and thalamic-derived) (Hisanaga and Sharp, 1990; Chapter 3, 4). 3) Moreover, *in vivo* studies indicate that the cells at this age (in the perinatal period) seem to require target innervation to prevent PCD (see Chapter 3). 4) Lastly, part of the survival regulation mechanisms may involve TrkB signaling as some small increases in cell death in certain thalamic nuclei were found in *trkB* knockout mice (see Chapter 3). Therefore, the mechanism regulating thalamic survival may be complex but is likely to involve at least neurotrophin signaling and activity regulation.

2. Thalamic explants and neurotrophin signaling

It was not known whether, and if so when, thalamic cells require Trk signaling for their survival. In Chapter 3, Lotto and I have shown that thalamic neurons develop a requirement for BDNF as they age. In addition, early thalamic neurons in cultures do respond to neurotrophins as early as E15 (Lotto et al., 1997) and become more dependent on them at E18-E19. However, in *in vivo* studies, there is no increase in cell death in the early (before E15) thalamus of the mutants. Thus, it is possible that 1) the Trks are not essential for thalamic survival at these early ages

or 2) there may be a trophic compensatory mechanism, e.g. trophic redundancy, that keeps the mutant thalamic cells surviving normally at the early stages. According to the findings in this chapter that no increased cell death is found in E15 thalamic explants of the mutants compared with the wild types, it is yet more possible that TrkB and TrkC signaling are not required for survival of the young thalamus.

As discussed earlier, the mechanisms regulating thalamic survival may be complex and I tested if TrkB or TrkC signaling alone is crucial for the thalamus in explants at E19. Unfortunately, the viability of all the wild type and the mutant thalami were low (<10%) with high values of standard error, preventing me from observing any possible increase in cell death in the mutants. It is possible that the surviving cells are a population of neurotrophin independent cells while the dead cells (in both wild types and mutants) are TrkB/TrkC dependent cells. If this is the case, a difference in thalamic survival between the wild types and the mutants cannot be observed by this experiment. I thus attempted to rescue the dead population that may require TrkB or TrkC signaling by adding K^+, since K^+ has previously been shown to depolarise and promote survival of various types of neuronal populations (including thalamic cells) (Ghosh et al., 1994; Magowan and Price, 1996; Limpscombe et al., 1988). However, interpretation must be made with caution. I predicted that if there is any difference in viability among the mutant and wild type cells, thalamic survival may require one or more of either the independent actions of TrkB or TrkC and K^+ depolarisation as K^+ itself might also enhance production of trophic factors.

3. The role of K^+ and neurotrophin signaling in the survival of thalamic explants

When KCl was applied to the thalamic explants of the mutants and the wild types, the survival of all thalamic explants increased markedly. Surprisingly, the E19 *trkB-/-* thalamic explants with added K^+ did not show any significant increase in cell death as found in *in vivo* studies (see Chapter 3). This also suggested that the survival promoting effects of K^+ on thalamic cells might not involve TrkB signaling which is opposite to what is found in cortical neurons. Ghosh et al (1994) showed that the

increase in cortical neuron survival after treatment with KCl was dependent on BDNF function. Anti-BDNF completely prevented the survival effects of KCl (Ghosh et al., 1994). They also further suggested that the survival promoting effects of KCl, via BDNF, involve the influx of Ca^{++} and intracellular Ca^{++} signaling. Jiang and Guroff (1997) also confirmed that BDNF promotes cell survival by signaling through intracellular Ca^{++}. In fact, however, the role of intracellular Ca^{++} signaling in preventing neuronal death is still unclear, because intracellular Ca^{++} is also thought to be involved in the PCD process (Pettman and Henderson, 1998).

One possible explanation as to why I did not observe a change in cell survival of the mutant thalami in the presence of K^+ may because K^+ depolarisation has a more generalised effect on regulating thalamic survival and does not act solely via neurotrophin signaling. It is also possible that K^+ may promote the release of more than one trophic factor (e.g. more than one neurotrophins and/or neurotransmitters) (Ben-Ari et al., 1994; Bessho et al., 1994). Therefore, thalamic cells *in vitro* (of either wild type or mutant), with the presence of K^+, may bypass their exclusive requirement for TrkB signaling *in vivo*. However, there is still no clear evidence to support these possibilities and they remain to be further investigated. In addition, although less likely, it is also possible that due to unknown congenital compensatory mechanisms present in the knockouts, thalamic explants of these animals respond to K^+ normally.

Chapter 6: Programmed cell death in *small eye* brain

Introduction

Pax-6 is a gene encoding a transcription factor with both paired- and homeo-domain DNA binding motifs (Walther and Gruss, 1991). Both motifs are highly conserved among species (reviewed by Callaerts et al., 1997). The gene is believed to play an essential role in the development of nervous system, eye, and nose. In the forebrain, *pax-6* mRNA is first detected at around E9 (Mastick et al., 1997) and is expressed restrictively in spatiotemporal patterns early during development in alar plate of the diencephalon and the telencephalon (Stoykova et al., 1996). As the animal develops, the expression of this gene in the forebrain is found in cerebral cortex, ventral thalamus (i.e. reticular nucleus, zona incerta and ventral lateral geniculate nucleus) and hypothalamus (Stoykova et al., 1996). The function of the gene is not only important to the cells that express it but also to other *pax-6* non-expressing tissues such as the dorsal thalamus. Recent studies have shown that proper formation and guidance of thalamocortical fibers requires *pax-6* (Kawano et al., 1999; Edgar, 1998).

Mice and rats with a natural point mutation in the *pax-6* gene, called *small eye* (*Sey*), have severe malformations in many developing tissues (Hill et al., 1991; Kawano et al., 1999). The homozygous mice (*Sey/Sey*) lack eyes and nasal cavities. The brains are markedly reduced in size and show several defects in the diencephalon (Warren and Price, 1997). In addition, *Sey/Sey* mutants also have undeveloped olfactory bulbs (Hogan et al., 1986), defects in the posterior commissure and tract of the postoptic commissure (Mastick et al., 1997), and an absence of thalamocortical formation (Kawano et al., 1999). Migratory capacity is also altered in late-born cortical precursors (Caric et al., 1997). Due to these and other severe anomalies, *Sey/Sey* homozygotes cannot survive after birth (Gotz et al., 1998).

One explanation for the defects caused by the *pax-6* mutation could be that the gene normally has a regulatory role in the expression of cell adhesion molecules. For instance, cell migration and boundary formation defects occur as a result of alteration in R-cadherin and tenascin-C expression in *pax-6* mutant mice (Stoykova et al., 1997) and possible alteration of expression of TAG-1 and PSA-NCAM molecules (Fukuda et al., 2000). Moreover, the adhesion molecule L1, which is known to regulate axon guidance and fasciculation, can be activated by *pax-6* (Meech et al., 1999).

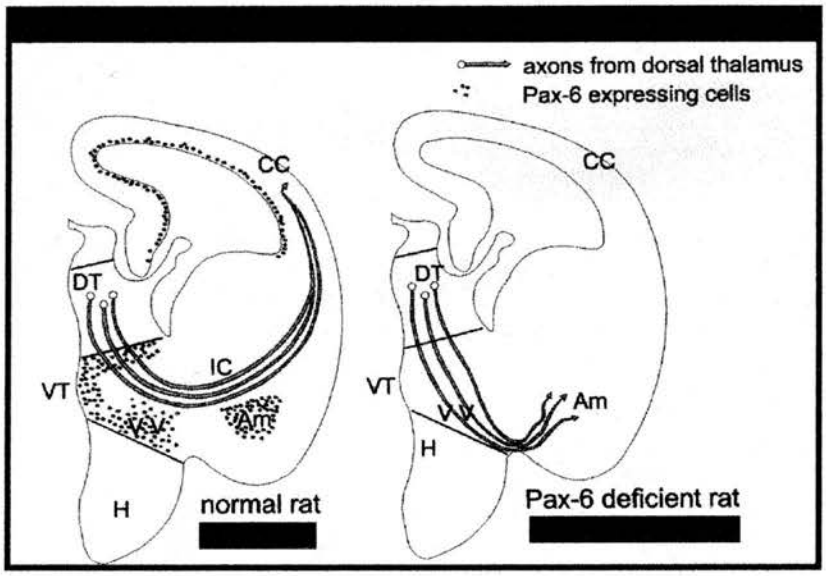
Aside from the possible role of *pax-6* in regulating cell adhesion molecules, some have proposed a role for the gene in regulating cell turnover. It has been shown previously that *pax-6* regulates cell proliferation. Cell numbers and percentages of BrdU incorporating cells (mitotic cells) were markedly reduced in both the diencephalon and the telencephalon of the mutant brain (Warren and Price, 1997; Gotz et al., 1998; Warren et al., 1999). However, it has not been clearly demonstrated whether *pax-6* has a direct or indirect role in controlling cell death. Gotz et al. (1998) showed that, at E13.5, there is no significant change in the rate of cell death in the mutant cortex. Neither was there a difference in cell death between *Sey/Sey* and wild type in developing diencephalon from E11 to E15 (Warren and Price, 1997). However, in the *Sey/Sey* mutant there is an absence of cell death which is normally present in lateral nasal processes at around E11 (Grindley et al., 1995). It is thought that *pax-6* has an indirect role in regulating cell death in nasal development.

In the dorsal thalamus of the mutant, thalamocortical fibers are disrupted (Figure 6.1). The Neurotrophic Hypothesis predicts that these fibers are required for thalamic survival. It is not known whether this hypothesis is true in the thalamocortical system, but it is possible that *pax-6* might cause secondary cell death in dorsal thalamus. In this study, I investigated the pattern of programmed cell death in *Sey/Sey* thalamus *in vivo* and determined the viability in culture of mutant thalamic cells in the presence or absence of diffusible trophic factors from the mutant or wild type target tissues (cerebral cortices). This was aimed at understanding whether the

gene and/or the establishment of thalamocortical fibers are essential to the viability of the thalamic cells.

Figure 6.1

A diagram showing thalamocortical formation in wild-type (left) and *Sey/Sey* (right) rat. The picture is taken from Kawano et al. (1999). The malformation pattern is also similar in mice (Edgar, 1998).


axons from dorsal thalamus
Pax-6 expressing cells
CC
DT
IC
VT
Am
H
normal rat
CC
DT
VT
Am
H
Pax-6 deficient rat

Materials and Methods

1. *In vivo* study

Apoptosis Labeling

E17 and E19 *Sey/Sey* (n = 5, n = 4) and Swiss wild type mice (n = 5 each) were dissected and processed histologically. *Small eye* homozygous mutant mice (*Sey/Sey*) were easily identified by the absence of eyes and a shortened snout. The TUNEL technique was carried out as described in Chapter 2.

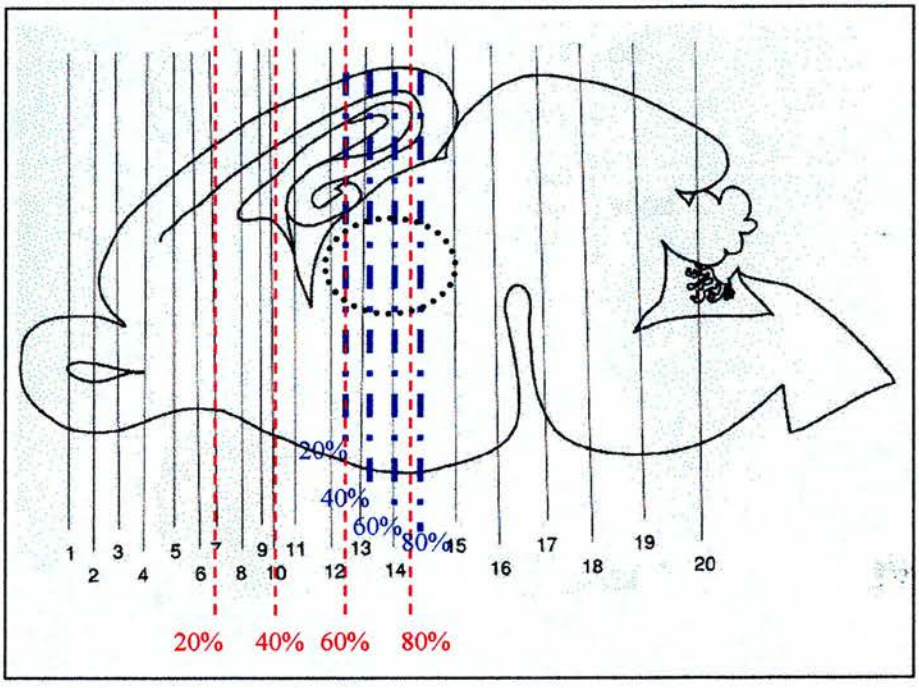
Identification of thalamic boundaries was done according to Schambra et al. (1992) and Paxinos et al. (1994) (see also Chapter 2). Dorsal thalamus was identified as a mass of cells lying between the external medullary lamina (eml) (distinguishing dorsal from ventral thalamus) and the fasciculus retroflexus (fr – hablenulo-peduncular tract) (distinguishing dorsally from epi-thalamus). In *Sey/Sey* embryos, the eml and fr are less clear but still identifiable as cell-sparse borders in the *Sey/Sey* diencephalon.

Cell counts were made in both the cerebral cortex and the thalamus. Details of using the grid under the 40x objective for counts were as described in Chapter 3. I counted the normalised proportions of TUNEL positive cells per 1000 cells in randomly selected areas. For cerebral cortex, counts were made in sections approximately every 20% of the distance from the most anterior section where the cortex was first seen to the most posterior section (see Figure 6.2, red lines). The average proportion of dead cells in cerebral cortex was calculated by dividing the total number of TUNEL positive cells in all areas (from section at 20%, 40%, 60%, and 80%) by the total number of cells counted in those areas and normalising to the proportion per 1000 cells.

Since the morphology of *Sey/Sey* dorsal thalamus is severely distorted and the thalamic nuclei could not be identified in sections, the division of the thalamus for cell counts was done in the same way as in the cerebral cortex. I counted in sections

Figure 6.2

A diagram showing how the developing mouse brains are divided and counted. Positions of the sections at 20%, 40%, 60%, and 80% of the distance through the cerebral cortex from anterior to posterior are illustrated by red dotted lines. Positions of the sections at 20%, 40%, 60%, and 80% of the distance through the thalamus from anterior to posterior are illustrated by blue dotted lines. Examples of sections of the dorsal thalamus used for cell counts in each are illustrated in Figure 6.3.


20%
40%
60%
80%
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
20%
40%
60%
80%

through the thalamus at 20%, 40%, 60%, and 80% of distance from where I first saw thalamus to the last sections in which I could observe it in coronal sections (see Figure 6.2, blue lines). The first section (20%) corresponded to anterior thalamic nuclei, the section at 40% was around the region of ventral nuclei and dorsomedial group, the section at 60% corresponded to ventral posterior and lateral geniculate nuclei, and the section at 80% corresponded to the area of posterior thalamic nuclei.

2. *In vitro* study

Dissociated cells were obtained as described earlier in Chapter 2 (total n = 45 wells). Although *Sey/Sey* brains showed severe abnormalities in the diencephalon, it was still possible to identify their dorsal thalamus at E15. The thin borders of two tracts (eml and fr) were used to distinguish the dorsal thalamus. Conditioned media were obtained from both wild type and *Sey/Sey* E19 cortices, with 40 pieces of cortex per 500µl culture medium in each well of 24 well culture plates. Details of this are described in Chapter 4.

3. Analysis

In both in vivo and in vitro studies, differences in cell death were tested statistically with Students' t-test.

Results

Coronal sections of the brain (stained with DAPI) around the region of the thalamus of E19 wild type and *small eye* brains are shown in Figure 6.3. It was clear that the *Sey/Sey* brain was markedly underdeveloped and was reduced in size (Figure 6.3 b, d, f, and h). The third ventricle was prominently enlarged. Dorsal thalamus was identified by two thin strips of cell sparse zones in the diencephalon as described in Methods, and the lower border was around the level of the upper closure of the third ventricle. Abnormal structure within the dorsal thalamus of the *Sey/Sey* mutant was evident. It was impossible to subdivide the dorsal thalamus or identify individual thalamic nuclei.

Dramatic increase in cell death is found in *Sey/Sey* thalamus at E19, but not at E17

A previous study reported that there were no differences in cell death between wild type and *Sey/Sey* mice aged from E11 to E15 (Warren and Price, 1997). This study effectively continues that previous study to older ages. At E17, some increase in cell death was found in the first anterior sections (20%) of the thalamus (area corresponding to paraventricular or anterior nuclei) with an average of 7.44 cells/1000 cells in *Sey/Sey* mice. However, the standard deviation is high ($\pm$ 4.45 cells/1000 cells) and the cell death rate was not significantly different to wild-types (1.44 $\pm$ 0.69 cells/1000 cells). Cell death in other regions in *Sey/Sey* mutants was as follows: 1.15, 0.84 and 1.64 cells/1000 cells (corresponding to areas 40%, 60%, and 80% from anterior to posterior through the thalamus). The cell death rates in sections in the wild types were 0.8, 1.2, and 2.9 cells/1000 cells. The average cell death rate throughout all sections was not different in the mutant (1.45 $\pm$ 0.53 cells/1000 cells in wild type, and 2.54 $\pm$ 0.96 cells/1000 cells in *Sey/Sey*). A summary of these cell counts is shown in Figure 6.4a.

At E19, the proportion of apoptotic cells rose sharply in *Sey/Sey* thalamus, with the average for total thalamic cell death being 53.29 $\pm$ 10.43 cells/1000 cells,

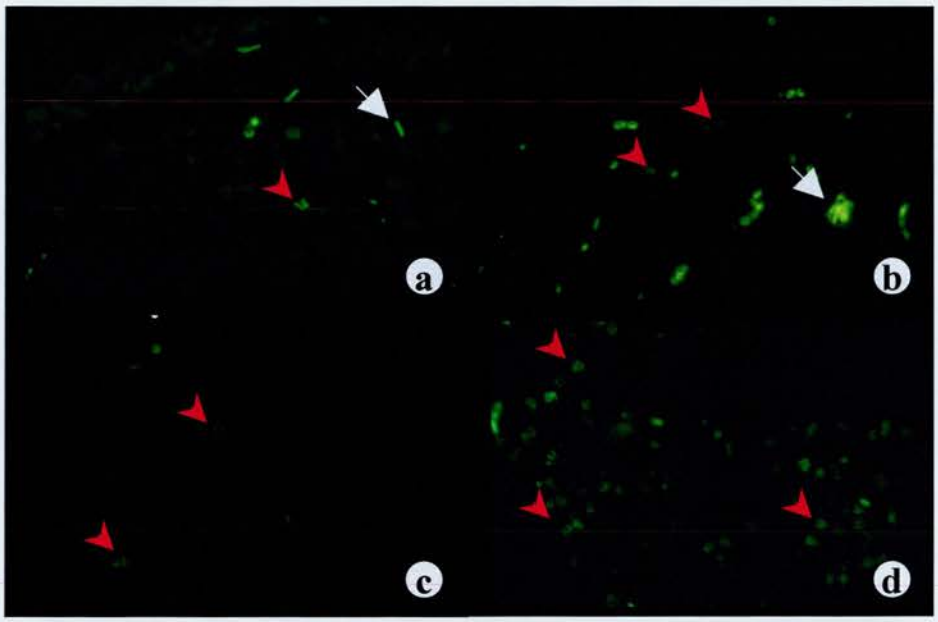
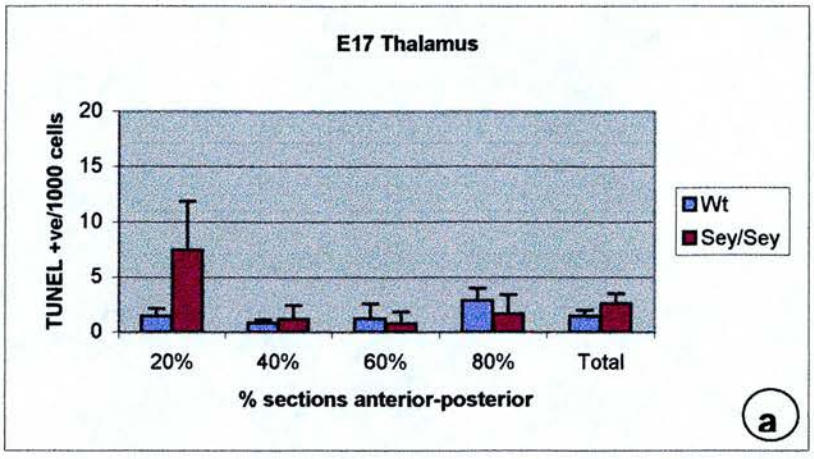

a
b
c
d
100μm

Figure 6.5

Photomicrographs showing TUNEL positive cells in representative areas of E19 dorsal thalamus in coronal sections. An increase in the numbers of TUNEL positive cells can be found in the section of the anterior (20%) *Sey/Sey* thalamus (b), compared with the wild type thalamus (a). In a posterior section (section at 60%) of the dorsal thalamus, a marked increase in cell death was found in the mutant (d), compared to the wild type thalamus (c). Red arrowheads depict TUNEL positive cells. White arrows = false-positive cells.


E17 Thalamus
TUNEL +ve/1000 cells
20
15
10
5
0
20%
40%
60%
80%
Total
% sections anterior-posterior
Wt
Sey/Sey
a

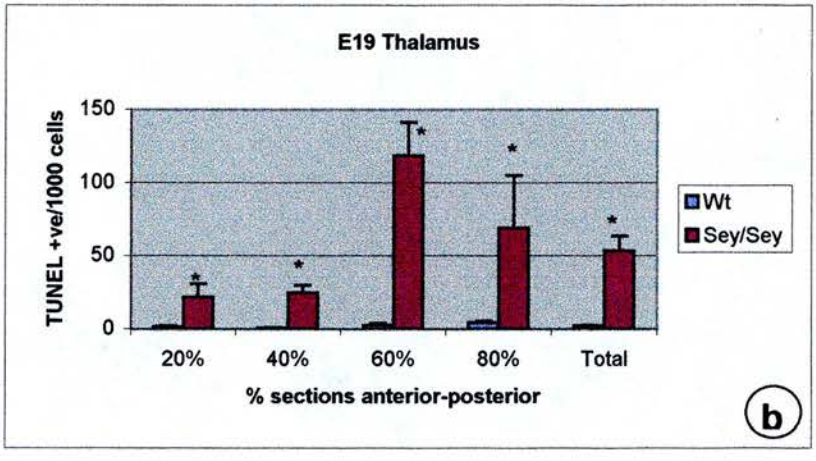

E19 Thalamus
TUNEL +ve/1000 cells
150
100
50
0
20%
40%
60%
80%
Total
% sections anterior-posterior
Wt
Sey/Sey
b

Figure 6.4

Graph showing the number of TUNEL positive cells per 1000 cells in E17 (a) and E19 (b) thalamus of the *Sey/Sey* (red bars) and wild type (blue bars). Note: in (a), the increase in average cell death in the rostral section (20%) is not significant. In (b), a marked increase in cell death is found in every section of the mutant thalamus, particularly at the posterior ends of the sections.

Figure 6.3

Morphological defects in the *Sey/Sey* dorsal thalamus stained with DAPI. (a), (c), (e) and (g) are E19 wild type thalamus. (b), (d), (f) and (h) are *Sey/Sey* thalamus. Pictures are in anterior-posterior order (sections at 20% are (a) and (b), 40% are (c) and (d), 60% are (e) and (f), and 80% are g) and h)). Two thin strips of cell-sparse areas (dotted lines) determine the upper and lower borders of the dorsal thalamus in wild type and *Sey/Sey* brains. Note: the mutant thalamus is smaller and distorted in shape. Thalamic nuclei are indistinguishable. (Pictures of E19 wild type brains are the same as shown in Figure 2.1, Chapter 2).

while the average cell death in wild type thalamus was only 2.0 ± 0.4 cells/1000 cells ($p < 10^{-4}$) (Figure 6.4b, Figure 6.5). The anterior half of the *Sey/Sey* thalamus had much lower death rates than the other half. Cell death rates in sections at 20% and 40% (anterior) were 21.71 ± 9.2 and 24.8 ± 5.0 cells/1000 cells, compared with sections at 60% and 80% (posterior) which gave rates of 118.5 ± 22.68 and 68.91 ± 35.93 cells/1000 cells ($p<10^{-3}$), while cell death rates in wild types at E19 were low with the values ranging from 0.86 to 5.48 cells/1000 cells (from section 20% to 80%) (Figure 6.4b, Figure 6.5).

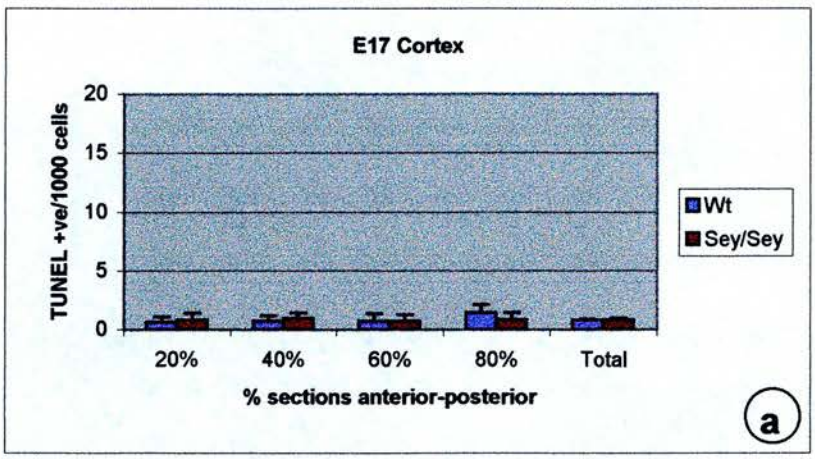
It should be noted here that 1) the increase in cell death in *Sey/Sey* thalamus at E19 (approximately 21-118 cells/1000 cells) was even higher than the peak cell death which occurs in normal thalamus at P1 (4-50 cells/1000 cells, see Chapter 3). 2) Although the thalamic nuclei were unidentifiable, the area that showed the highest cell death in the mutant (~60% posterior), was around the same level as ventral posterior nuclei in wild type thalamus, which showed the highest death rates compared to other thalamic nuclei at P1.

Cortical cell death in the *pax-6* mutants is not affected

To further confirm that an increased cell death in the mutant thalamus at E19 was not a reflection of generally increased non-specific cell death, I also investigated cell death in the cortex of these mice. The cerebral cortex of the mutant was thinner and the cells were more densely packed than in the wild types (as previously shown by Caric et al., 1997; Mastick, et al., 1997; Warren and Price, 1997; Warren et al., 1999). A TUNEL study in E17 and E19 cortices revealed no significant difference in death rates in the mutants (Figure 6.6a, b). The total cell death count per 1,000 cells in E17 *Sey/Sey* cortices remained low (0.84 ± 0.12 cells/1000 cells) which was similar to that in wild types (0.84 ± 0.06 cells/1000 cells). There was no difference in cell death in any particular area from anterior to posterior in wild types (0.66, 0.71, 0.70, 1.46 cells/1000 cells) compared to *Sey/Sey* embryos (0.82, 0.93, 0.74, 0.86 cells/1000cells). Likewise, E19 *Sey/Sey* cortices had an average cell death of 1 ± 0.22 cells/1000 cells, around the same rate as wild-types (0.91 ± 0.21 cells/1000 cells). The distributions of cell death at both ages in both *Sey/Sey* and wild type were

Figure 6.6

Graphs show cell death ratio in E17 (a) and E19 (b) mutant (red bars) and wild type (blue bars) cerebral cortex. No significant difference in cell death was found in any region of the cortex in either age.


E17 Cortex
TUNEL +ve/1000 cells
20
15
10
5
0
20%
40%
60%
80%
Total
% sections anterior-posterior
Wt
Sey/Sey
a

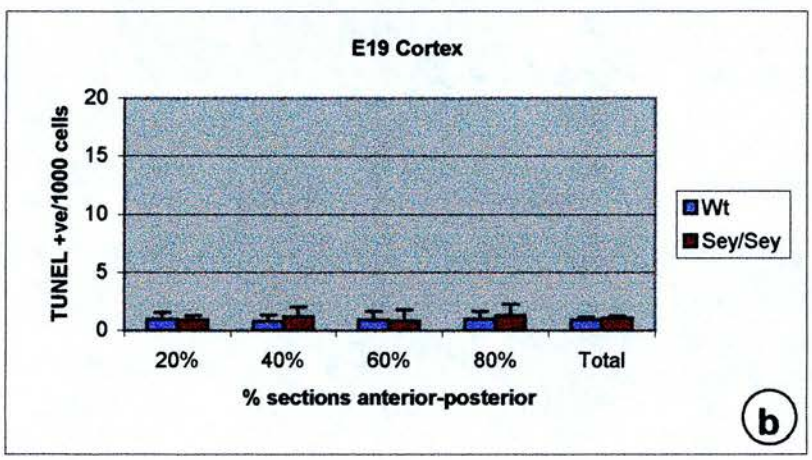
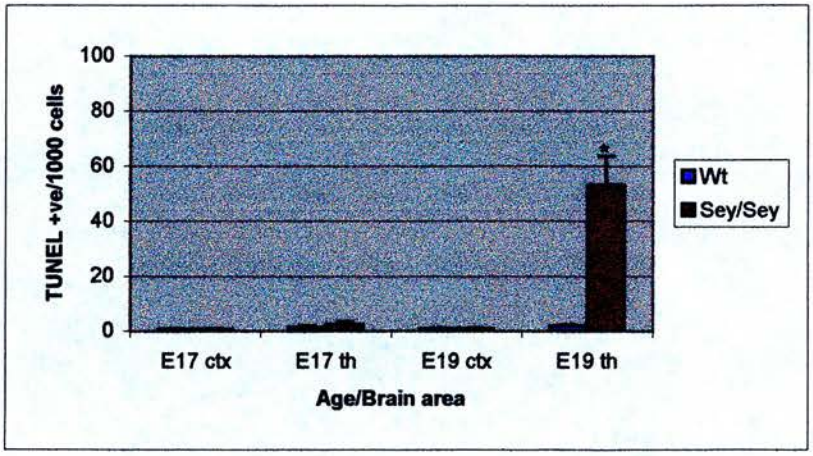

E19 Cortex
TUNEL +ve/1000 cells
20
15
10
5
0
20%
40%
60%
80%
Total
% sections anterior-posterior
Wt
Sey/Sey
b

Figure 6.7

A summary graph comparing averages of total cell death in both thalamus and cortex of the E17 and E19 *Sey/Sey* and wild type mice. Note: only *Sey/Sey* E19 thalamus has a marked increase in cell death.


100
80
60
40
20
0
TUNEL +ve/1000 cells
E17 ctx
E17 th
E19 ctx
E19 th
Age/Brain area
Wt
Sey/Sey

scattered. Anterior-posterior distributions of cell death were similar (see summary in Figure 6.4a, b).

A summary of the *in vivo* TUNEL studies in the whole thalamus and cortex is also shown in Figure 6.7.

In vitro experiments

Cell culture experiments were done to investigate the viability of *Sey/Sey* thalamic cells, their response to trophic factors obtained by conditioning their medium with cerebral cortex, and the production of target derived trophic factors by the *Sey/Sey* cortex. These were aimed at identifying the possible mechanisms by which the mutated *pax-6* gene causes increased thalamic cell death *in vivo*. Possible mechanisms include the following: 1) A direct effect of the *pax-6* mutation, causing thalamic cell death before and/or after the period of target innervation. Although this possibility is the least likely (see discussion), cell culture studies would help to exclude it. 2) Reduction in the trophic supply by the target cerebral cortex. This was tested by observing the ability of *Sey* CCM to promote thalamic survival in vitro. 3) Thalamocortical disruption inhibiting thalamic cells from obtaining sufficient trophic factors. This could be inferred if *Sey/Sey* thalamic cells survive normally with wild-type CCM and if *Sey* CCM produces the trophic effect of wild-type CCM.

In a first set of culture experiments, I cultured wild type and *Sey/Sey* thalamic cells for 5 days. Comparing the viability of *Sey/Sey* and wild type thalamic neurons showed similar patterns (Figure 6.8a). Up to 50% of both *Sey/Sey* mutant and wild type thalamic cells survived for two days. This indicated that the ability of thalamic neurons to survive is not directly affected by the *pax-6* mutation. It is interesting that survival of *Sey/Sey* thalamus seemed better than that of wild type thalamus after 3 days of culture (28% in 3-day and 14% in 4-day culture, compared to 18% at 3 days and only 2% at 4 days for wild-type cells). I cannot fully explain this result but it is possible that the mutation in *pax-6* makes thalamic cells less susceptible to death at this age *in vitro*. At 5 DIV (~P0 *in vivo*), on the other hand, very few cells survived

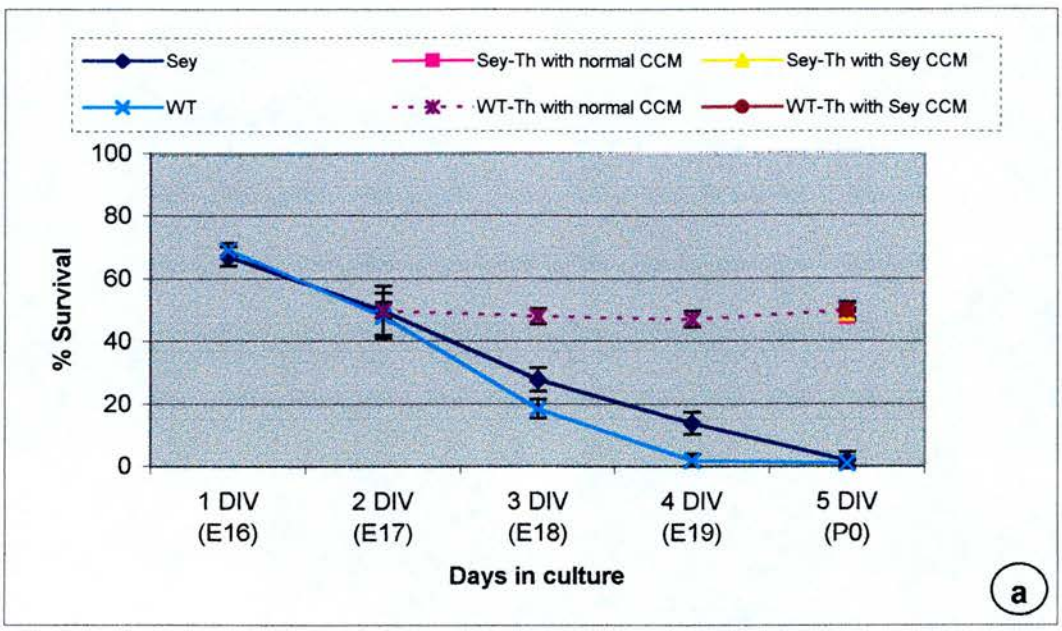
Figure 6.8

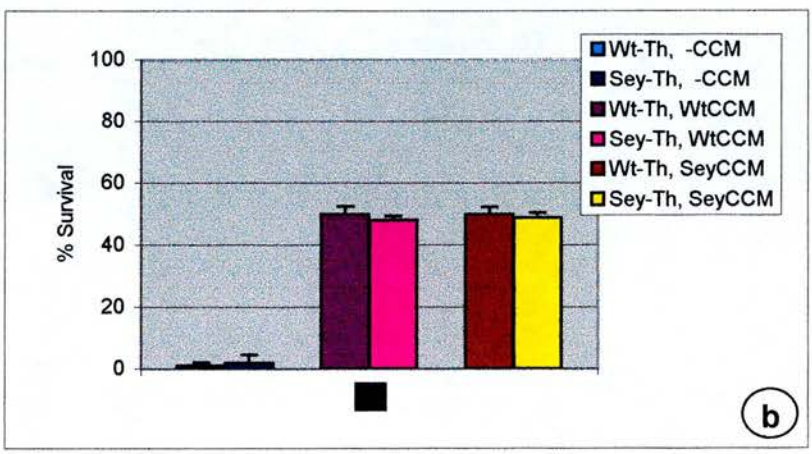
Graphs showing thalamic viability in cultures

(a) Survival percentages of *Sey/Sey* thalamic cells comparing to the wild-type cells when cultured for 5 days. *Sey/Sey* thalamic viability (blue) is similar to the wild type (light blue). With the supply of either *Sey/Sey* CCM or wild-type CCM, *Sey/Sey* thalamic cells at 5^{th} day respond and survive normally (pink and yellow lines, comparing to the wild type viability – purple and brown lines).

(b) A more clear demonstration of the thalamic viability at the 5^{th} day of culture: Survival of both mutant and wild type thalamic cells is very poor without the supply of CCM. *Sey* CCM promotes wild type thalamic cell survival to the same extent as wild-type CCM. Survival of *Sey/Sey* thalamic cells is equal to the wild type viability with or without CCM.

(CCM = E19 cortical conditioned medium, "-CCM" = without CCM, *Sey* = *Sey/Sey* tissue, Th = thalamic cells, WT = Wild-type)


Sey
Sey-Th with normal CCM
Sey-Th with Sey CCM
WT
WT-Th with normal CCM
WT-Th with Sey CCM
100
80
60
40
20
0
% Survival
1 DIV (E16)
2 DIV (E17)
3 DIV (E18)
4 DIV (E19)
5 DIV (P0)
Days in culture
a


Wt-Th, -CCM
Sey-Th, -CCM
Wt-Th, WtCCM
Sey-Th, WtCCM
Wt-Th, SeyCCM
Sey-Th, SeyCCM
100
80
60
40
20
0
% Survival
b

in both mutants and wild types. This suggested that the cells in both wild types and the mutants require external trophic support as shown previously in Chapters 3 and 4.

Next, I tested two hypotheses: 1) whether *Sey/Sey* thalamic cells were able to respond normally to target derived factors from the cortical conditioned medium and 2) whether the increased cell death in *Sey/Sey* thalamus might be due to the reduction in the production of trophic factors in the cortex. These were done by culturing wild type or *Sey/Sey* thalamic cells for five days in the presence of conditioned medium obtained from either *Sey/Sey* or wild type E19 cortex (E19 *Sey* CCM or E19 Wt CCM) (Figure 6.8a, b). The results showed that: 1) *Sey/Sey* thalamic cells responded to trophic substance(s) from E19 *Sey* CCM (48 $\pm$ 1.5%) and normal E19 CCM (49 $\pm$ 1.7 %) as normally as wild type cells did and 2) E19 *Sey* CCM had the same level of survival promoting effects as normal E19 CCM on 5 DIV wild type thalamic cells (the cells had nearly 50% survival).

From these *in vitro* results, it is likely that the *pax-6* gene does not have a direct role in either regulation of thalamic cell death or the production of trophic factors released from the cortex.

Discussion

Several striking anomalies including a reduction in tissue size in many CNS regions are found in the *pax-6* mutant brain. It is possible that the *pax-6* mutation affects either cell proliferation, cell interaction and guidance, cell migration, cell survival, or a combination of these processes. Reductions in proliferation and cell number revealed by BrdU studies are found in many CNS regions such as diencephalon and cerebral cortex (Caric et al., 1997; Warren and Price, 1997). Several axonal pathways are disrupted. These include posterior commissural fibers, the postoptic commissural tract, olfactory fibers and thalamocortical fibers (Mastick et al., 1997; Grindley et al., 1995; Kawano et al., 1999). There are also migratory defects during neocortical development, i.e. some cortical cells of the mutant which would normally disperse and migrate form clusters in the subventricular zone. The possible downstream targets of *Pax-6* which regulate cell migration include cell adhesion molecules such as L1, PSA-NCAM and others (Caric et al., 1997; Schmahl et al., 1993). However, to date, there is not enough evidence to suggest whether defects in *pax-6* directly control cell survival.

In fact, when I studied the possible abnormality in cell death in the mutant, I first looked at the cell death in cortical tissue before examining the thalamus. The rate of cell death in the cortex was relatively normal at both ages studied (E17 and E19). This agrees with the cell death studies done at E13-E14 in cortex by Gotz et al., (1994), where they also found no significant difference in the rate of cell death in mutants. Recent studies also confirmed that there was no change in cell death in the later stages of developing mutant cortex (Fukuda et al., 2000). Altogether, these findings suggested that cell death is not directly controlled by *Pax-6* itself or by immediate downstream mechanisms controlled by *Pax-6* (i.e. the *pax-6* mutation is not directly lethal to developing cells). However, as will be discussed below, it is still possible that *Pax-6* alters some environmental factors that older (but not younger) thalamic cells require to survive and thus results in an increased thalamic PCD only at the later stages of development (my speculation is that such environmental factors include the disruption of thalamocortical fibers).

Similar to events in cortex, early embryonic *Sey/Sey* thalamic cells (E10-E15) do not show any striking abnormality of cell death (Warren & Price, 1997). My results also showed no obvious difference in PCD in E17 *Sey/Sey* thalamus. However, surprisingly, the change in the cell death rate in E19 *Sey/Sey* thalamus was dramatic. Several possible explanations arise following this finding.

Possible direct survival role of *pax-6*?

Since *Sey/Sey* mice die soon after birth, it is possible that the increased cell death found in the late stages of thalamic development is due to nonspecific cell death and/or *Sey/Sey* mice might have already died before they were actually born. However, the *Sey/Sey* embryos I obtained had no apparent signs of this (embryos seemed to be still viable at the time of removal). The increase in PCD found in the *Sey/Sey* E19 brain was regional even within the dorsal thalamus itself (i.e. with a posterio-anterior gradient). I concluded that this rise in cell death in the mutant thalamus is a specific process.

Several findings do not favor the hypothesis that *pax-6* has a direct role in regulating cell survival. First of all, there was no increase in cell death in the mutant cerebral cortex, where *pax-6* mRNA is normally expressed. No other areas of E17 or E19 brains had as high number of TUNEL labeled cells as the dorsal thalamus of E19 mutants (no quantitative data, but I observed the appearance in the distribution of TUNEL positive cells compared with other adjacent brain areas). Secondly, *Pax-6* is an early developmental transcription factor, and overall morphological defects observed in the mutant appear as early as E9 (Walther and Gruss, 1991; Puelles and Rubenstein, 1993, Mastick et al., 1997) while the increased thalamic cell death was observed at the perinatal period. Moreover, *in vitro* 5-day culture of dissociated E15 mutant thalamic cells showed the same survival pattern as wild type E15 cells (i.e. young *Sey/Sey* thalamic cells survive normally).

Since *pax-6* expression shows spatio-temporal changes in pattern and alters with time, it is possible that *Pax-6* affects particular gene(s) at specific ages, e.g.

genes that express in the thalamus only at E19, which is(are) required for thalamic survival. This possibility is unlikely because when *Sey/Sey* thalamic cells in 5-day culture (around P0 in vivo) were supplied with medium conditioned by either wild type or *Sey/Sey* cortex, their survival rate appeared normal. This means that intrinsic thalamic viability is not altered by *pax-6* mutation as the cells mature.

Cell death in E19 *Sey/Sey* thalamus is an indirect consequence of the *pax-6* mutation?

According to the Neurotrophic Hypothesis, target derived trophic support and the ability of neurons to establish proper connections are thought to be essential for survival. Previous studies have also shown the importance of target derived factors in the maintenance of thalamic survival particularly during the period of target innervation – E17 to early postnatal days (Price et al., 1995; see also in Chapter 3, 4). At E19, thalamic cells undergo many changes in their biological activity and their physiological properties. Unlike the E17 cells, they are more vulnerable and subject to death *in vitro*. They become more dependent on the supply of trophic factors from cortical conditioned medium and are highly sensitive to exogenous K^+ stimulation, most likely via an activity dependent mechanism (Magowan and Price, 1997). I tested if in these *Sey/Sey* animals, the cerebral cortex produced less trophic support to the thalamus, which could explain the striking PCD phenomenon at this age *in vivo*. However, *Sey/Sey* cortical conditioned medium had equivalent effects to the wild type cortical conditioned medium when tested on both wild type and *Sey/Sey* thalamic cells in cultures. Furthermore, *Sey/Sey* thalamic cells responded to cortical trophic factors normally. This implied that trophic factors released from the cortex are not deprived in the mutant. Then, the question remains, why do more *Sey/Sey* thalamic cells die at E19?

The answer seems to be that the path by which thalamic cells obtain trophic factors is absent. Coinciding with the time at which an increased PCD is found in the mutant thalamus, E19 thalamic cells become dependent on their target as shown in both *in vitro* and *in vivo*. The thalamocortical system in the *Sey/Sey* embryo is malformed (Kawano et al., 1999; Edgar, 1998). Mastick et al. (1997) showed that

Pax-6 is essential for navigation of axons of many CNS populations. The thalamocortical fibers which are normally guided by the expression of *pax-6* are misdirected at the ventral thalamus and terminate at the wrong target, the amygdala (Kawano et al., 1999). They never reach the cortex. DiI labeling in the thalamus also indicated the absence of thalamocortical axons from the telecephalon (Edgar, 1998).

Unfortunately, the *pax-6* mutants did not survive after birth. This prevented me from carrying out further studies on the consequences of thalamocortical disruption and thus I could not observe the effect of the mutation on the peak period of thalamic PCD, which normally occurs at P1. In addition, the culture of the older thalamic cells is much more difficult to manipulate so that the study of this mutant was focused only on the period before birth.

In summary, my results and the information from previous axonal formation studies strongly implicate thalamocortical fibers, rather than *pax-6* itself, in the survival of thalamic cells during the critical period of development.

Interestingly, it should be noted from the studies in *trkB* knockouts and *pax-6* mutants that the increase in thalamic cell death in both cases occurs prenatally, during the time when the thalamocortical axons have already started to navigate, but before the natural peak of PCD at P1. Combined with the observations on the time of the trophic requirement of the *in vitro* E17-E18 cells, this suggests that the process that makes thalamic cells become dependent on trophic factors occurs before the period of naturally occurring cell death. The time of thalamocortical innervation does not precisely coincide with the peak period of PCD but is an earlier event that may induce cells to commit to the trophic competition process.

Chapter 7: Study of cell proliferation in mice defective in TrkC receptor gene in thalamus and cortex

Introduction

Neurotrophin-3 (NT-3) is known to be important for neuronal and glial development (Kahn et al., 1999). Through TrkC, NT-3 signals a number of trophic effects, ranging from mitogenesis, through differentiation to promotion of survival, depending on the developmental stage of the target cells (see review by Chalazonitis, 1996). Both *NT-3* and *trkC* mRNA are expressed from around midgestation in several CNS regions including thalamus and cortex (Barbacid et al., 1994). Via a Ras dependent pathway, they can induce cell proliferation, while via a SNT pathway (see Chapter 1), they induce cells to stop mitosis and promote cell differentiation (Ockel et al., 1996; Lewin and Barde, 1996; Segal and Greenberg, 1996). The regulation of NT-3 action is still unclear. In oligodendrocytes, NT-3 and TrkC are thought to play a major role in cell proliferation (Kahn et al., 1999). For neural crest cells, they promote both proliferation and differentiation (Hapner et al., 1998; Pinco et al., 1993; Kalcheim, 1992), but for cortical neurons, they reduce proliferation and increase cell differentiation (Ghosh and Greenberg, 1995; Pappas and Parnavelas, 1997). Findings in the sympathetic neuroblast population are controversial. Lu et al. (1996) reported that NT-3 had no effects on sympathetic neuroblast mitosis and Verdi and Anderson (1994) showed that it produced a mitogenic stop signal, while DiCicco-Bloom et al. (1993) showed that NT-3 promoted mitosis and induced both mitotic and non-mitotic cells to differentiate at the same time. In cortex and thalamus, there is no clear evidence as to whether NT-3 or TrkC signaling are essential to neuronal or glial proliferation/differentiation.

Concerning the development of the cerebral cortex, cortical cells are derived from neuroepithelial precursors that lie in a layer called the ventricular zone (VZ). Axons grow over this zone to form the intermediate zone (IZ). The first set of postmitotic neurons migrates through the IZ to form the preplate (PP). Cells in the

VZ also give rise to cells in the subventricular zone (SV). Later, the second generation postmitotic neurons migrates out of the VZ to form the cortical plate (CP) which splits the PP into the outer part – the marginal zone (MZ, which later become the cortical layer I in adult) and the inner part – the subplate (SP). The CP neurons then migrate along radial glial processes and form cortical layers II-VI in an 'inside-out' sequence (where layer VI cells are born first) (reviewed by Hatten, 1999; Parnavelas, 2000). Early born cells give rise mostly to neurons. Glial cells in the cerebral cortex were thought to be generated from the subventricular zone and appear after neurogenesis (Smart, 1972; Halliday and Cepko, 1992). In mice, extensive cortical gliogenesis can be found around E18-P0 and is thought to increase postnatally (Edgar, 1998; Levers, 1999). In adult, only cortical layers I-VI and white matter (which is derived from the IZ) are evident.

Like the cerebral cortex, the dorsal thalamus develops from germinal cells dividing in the ventricular zone. It emerges from the prosomere 2 (p2) region of the diencephalon at around E13 (Altman and Bayer, 1988). Unlike the CP, postmitotic thalamic cells do not form in an 'inside out' sequence. Instead, the early born thalamic neurons give rise to the most lateral thalamic nuclei and the late born cells give rise to the most medial nuclei (Altman and Bayer, 1979a; b; c; Angevine, 1970). In the mouse, the cells that will give rise to the thalamic nuclei are born between E11 and E15 (Angevine, 1970). Most cells generated around this period are neurons. From E15 to postnatal life the differentiation of functional thalamic nuclei becomes evident. However, the evidence for the period of gliogenesis in the thalamus is still obscure. If we accept the general hypothesis that gliogenesis often occurs after neurogenesis is complete (Smart, 1972; Halliday and Cepko, 1992), we might assume that it may occur any time after E15-E16.

Based on the evidence in Chapter 3, that there is a small reduction in cell death in young TrkC knockout thalamus (E13), I hypothesised that TrkC signaling may be required for homeostasis of cells during the period of cell proliferation in the developing brain. Since this is likely to directly affect the proliferating cells, we may be able to observe defects in knockout mice. In this study, therefore, I examined the

proliferation rate in the mutant brain from very early in neurogenesis at E11 to the time before birth by using BrdU immunocytochemistry.

5-bromo-2-deoxyuridine (BrdU) is a molecule that can be employed to study proliferation, migration and time of origin of cells in the developing central nervous system (Miller and Nowakowski, 1988). It is an alternative to traditional [^{3}H] thymidine labeling. Like [^{3}H] thymidine, BrdU is incorporated into the DNA of proliferating cells during S-phase of the cell cycle. These proliferating cells can be detected by immunohistochemistry (Miller and Nowakowski, 1988).

Materials and Methods

Animals and BrdU injections

Mice heterozygous for a targeted mutation in the *trkC* sequence encoding the catalytic domain of the TrkC tyrosine kinase receptor isoforms (Klein et al., 1994) were used. Heterozygous *trkC* were mated to give wild type, heterozygous, and homozygous mice. The pregnant mice were injected with a single dose (0.2mls of a 2mg/ml solution, i.p.) of BrdU dissolved in sterile saline on E11, E13, E15, E17, and E19. One hour later, the animals were sacrificed by cervical dislocation. Embryos were removed at the assigned stage of pregnancy. Parts of the embryos (either legs or tails) were removed for PCR genotyping (see Chapter 3). Brains were removed and processed histologically as described in Chapter 2.

Immunohistochemistry

Sections were treated with 0.1% trypsin in 0.1% $CaCl_2$ at 37°C, to permeabilise the cell membranes for 20 minutes. The sections were then washed with tris-buffered saline (TBS pH 7.6) for 5 minutes before being treated with 1 N HCl at 60°C for 8 minutes, to denature the double-stranded DNA molecules and expose the incorporated BrdU molecules. Sections were washed with TBS, preblocked with normal rabbit serum (NRS) in TBS (1:4 NRS:TBS) for 10 minutes. The sections were incubated with anti-BrdU (5µl/ml) for 30 minutes and then washed twice in TBS for 5 minutes each. A second preblocking with NRS followed. The sections were next treated with a biotinylated secondary antibody (5µl/ml) for 30 minutes). When this incubation was complete, the sections were once again washed with TBS, then treated with avidin and biotinylated complex kit (ABC kit, Vector) according to the manufacturer's instructions for 30 minutes. Then sections were washed and reacted with 3,3'-diaminobezidine (DAB, Sigma) solution (0.05% DAB, 750µl tris buffer, 20µl 0.1% H_2O_2 and one flake of imidazole) for 8 minutes, conterstained with cresyl violet and differentiated in graded concentrations of alcohol in water. Once dry, the sections were coverslipped with DPX mountant.

Summary of numbers of animals used in each set of experiments

	wild type	*trkC-/-*
E11	4	4
E13	3	4
E15	4	5
E17	4	3
E19	4	3

Cell counts and Analysis

I analysed two areas of the brain in this study: cerebral cortex and thalamus. In the cerebral cortex, four selected coronal sections (approximately equidistant, every 20% of the way through the brain) from the anterior to posterior were chosen for cell counts. Detail was as described in methods in Chapter 6 and Figure 6.1. For the thalamus, samples of major thalamic nuclei were chosen for cell counts in the same way as in Chapter 3.

A ratio of BrdU labeled cells over total cells in a selected area (under 40x magnification in a 10 x 10 grid-square bin, see detail in Chapter 3) is called 'Labeling Index' (Takahashi et al., 1991). I expressed this value as a percentage of BrdU labeled cells. Since a single BrdU dose labels nuclei in S-phase of the cell cycle only (Takahashi et al., 1991), the labeling indices (in percentages) obtained in this study represent the proportion of S-phase cells.

Both cortical and thalamic LIs in the mutant and the wild type brains were compared by Students' t-test.

Results

Thalamus

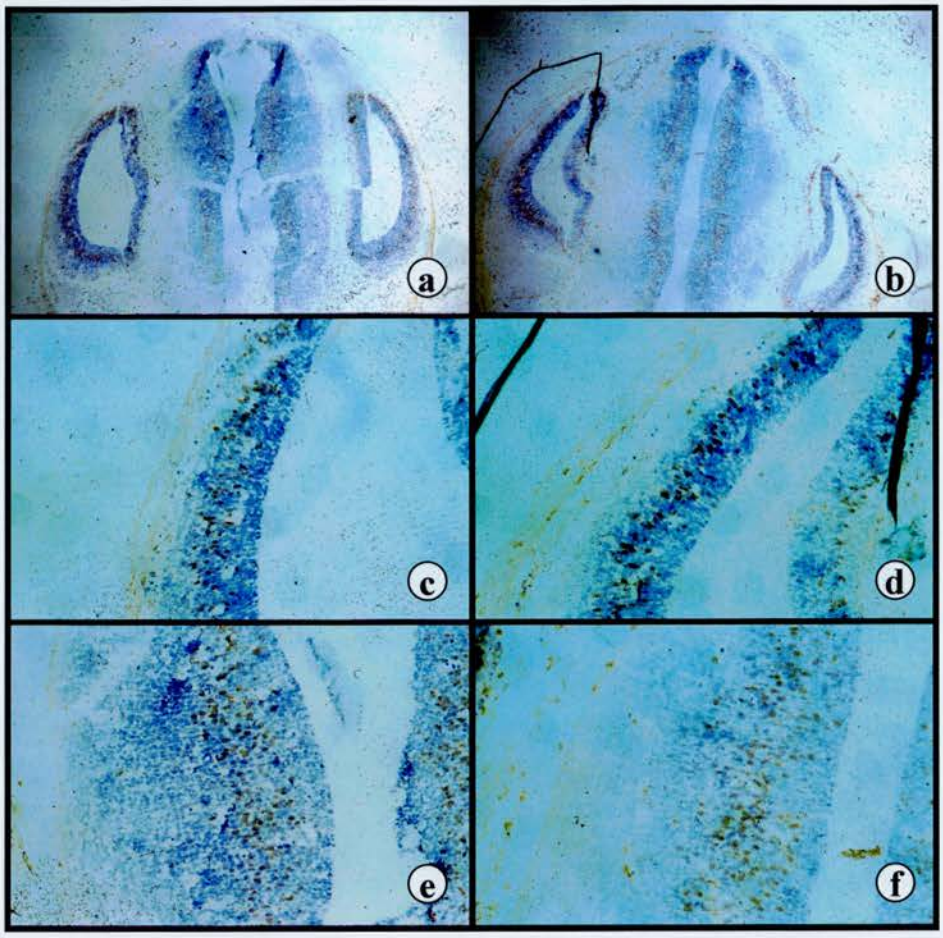
At E11 and E13, dorsal thalamus has not fully developed and so the analysis was made in the region of diencephalon which is likely to develop into the dorsal thalamus. Cell proliferation in mice at these ages is high. The rates of BrdU positive cells per total cells are 37.1 $\pm$ 3.17% in E11 and 19.09 $\pm$ 3.27% in E13 (Figure 7.1a, e, 7.2b, d). Most of the immunoreactive cells are localised nearer to the ventricular edge of the brain.

At E15, the dorsal thalamus and some of its nuclei can be identified. The percentages of BrdU labeled cells at this age were reduced markedly to 2.11 $\pm$ 1.49 % (see Figure 7.7b). However, in some brains, I still saw some highly proliferative regions particularly in the paraventricular area. This varied from one brain to another. This resulted in the high standard error in the counts in many nuclei and in the average for the thalamus (Figure 7.3b). From E17 to E19, the percentages of BrdU labeled cells were very low (less than 1%, 0.62 $\pm$ 0.13 % and 0.52 $\pm$ 0.3 % respectively). The distribution of the labeled cells was dispersed, and was no longer restricted to the ventricular/paraventricular area.

As in wild types, the *trkC-/-* thalamus decreased its proliferative rate markedly at around E15. I found no statistical difference between the LIs in the mutant thalamus and those in the wild type thalamus at any age, and in none of the thalamic nuclei studied (Figure 7.2b, 7.2d, 7.3b, 7.5b, 7.6b, and 7.7b for E11, E13, E15, E17, and E19). In more detail, the total average proliferative rates of *trkC-/-* thalamus were 33.85 $\pm$ 3.34% at E11, 21.24 $\pm$ 2.09% at E13, 2.13 $\pm$ 1.55% at E15, 0.44 $\pm$ 0.2 % at E17 and 0.49 $\pm$ 0.3 % at E19 (the proliferative rates for individual nuclei are summarised in the Figures).

Figure 7.1

Photomicrographs of E13 wild type and *trkC* mutant brains in coronal plane: low magnification picture of BrdU labeled cells (brown cells) in whole brain of (a) wild type, and in (b) *trkC-/-* embryos. Higher magnification of BrdU labeled cells in the cerebral cortex in wild type and mutant are shown in (c) and (d). Higher magnification of BrdU labeled cells in the wild type and the mutant thalamus are shown in (e) and (f).



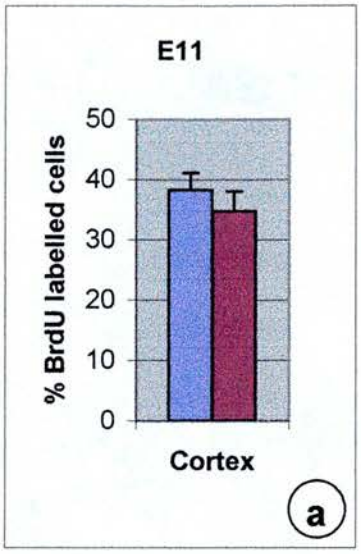
250μm (a, b)

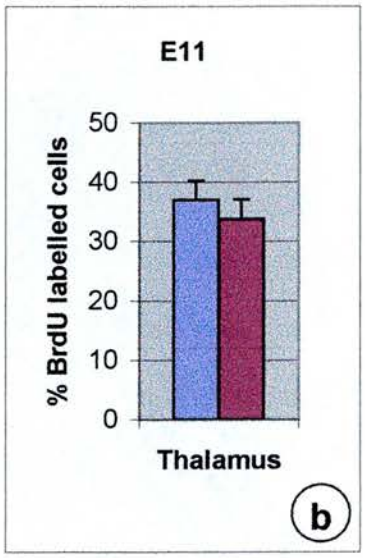
100μm (c-f)

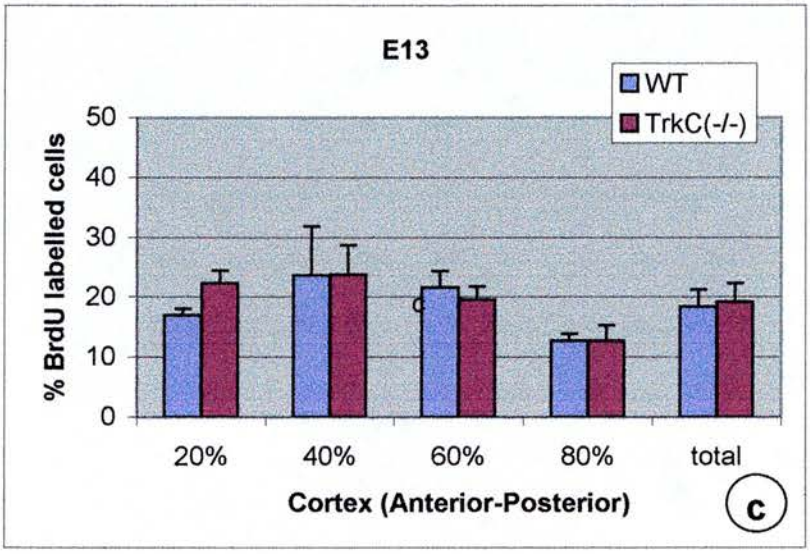
Figure 7.2

Graphs showing labeling index (percentage of BrdU labeled cells) in E11 and E13 cortex and thalamus of wild type and *trkC* knockout brains. (a) Shows BrdU labeling index in the E11 wild type and *trkC-/-* cerebral cortex. (b) Shows BrdU labeling index in the E11 wild type and *trkC-/-* thalamus. (c) Shows BrdU labeling index in the E13 wild type and *trkC-/-* cerebral cortex. Since the cortex has started to enlarge, cell counts are made in different areas anterior-posteriorly. (d) Shows BrdU labeling index in the E13 wild type and *trkC-/-* thalamus.

No significant difference in the labeling index is found at these ages.


E11
% BrdU labelled cells
50
40
30
20
10
0
Cortex
a


E11
% BrdU labelled cells
50
40
30
20
10
0
Thalamus
b


E13
WT
TrkC(-/-)
% BrdU labelled cells
50
40
30
20
10
0
20%
40%
60%
80%
total
Cortex (Anterior-Posterior)
c

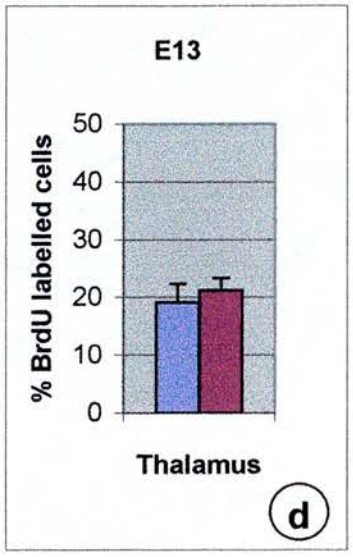
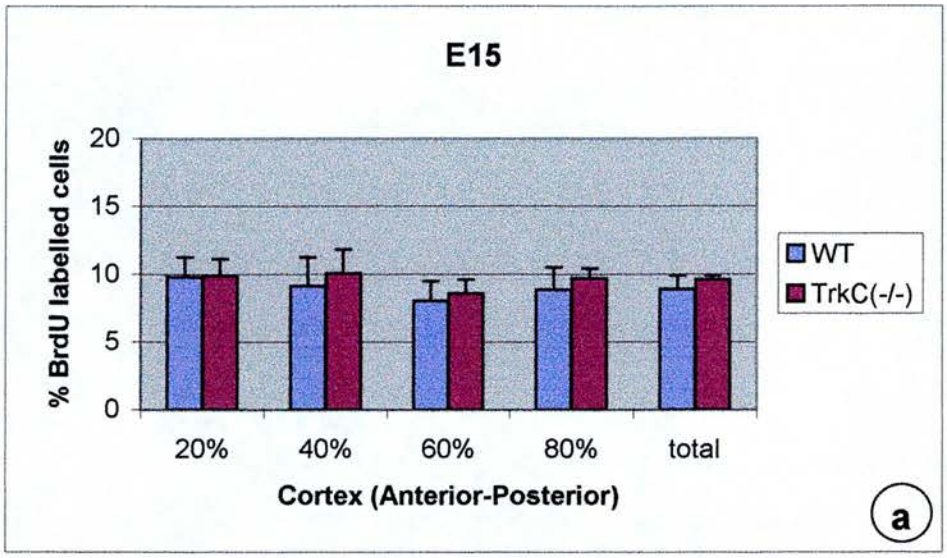

E13
% BrdU labelled cells
50
40
30
20
10
0
Thalamus
d

Figure 7.3

Graphs showing labeling index (percentage of BrdU labeled cells) in E15 cortex and thalamus of wild type and trkC knockout brains. (a) Shows BrdU labeling index in the E15 wild type and *trkC-/-* cerebral cortex. (b) Shows BrdU labeling index in the E15 wild type and *trkC-/-* thalamus. No significant difference in the labeling index is found in the mutants at this age.


E15
% BrdU labelled cells
20
15
10
5
0
20%
40%
60%
80%
total
Cortex (Anterior-Posterior)
WT
TrkC(-/-)
a

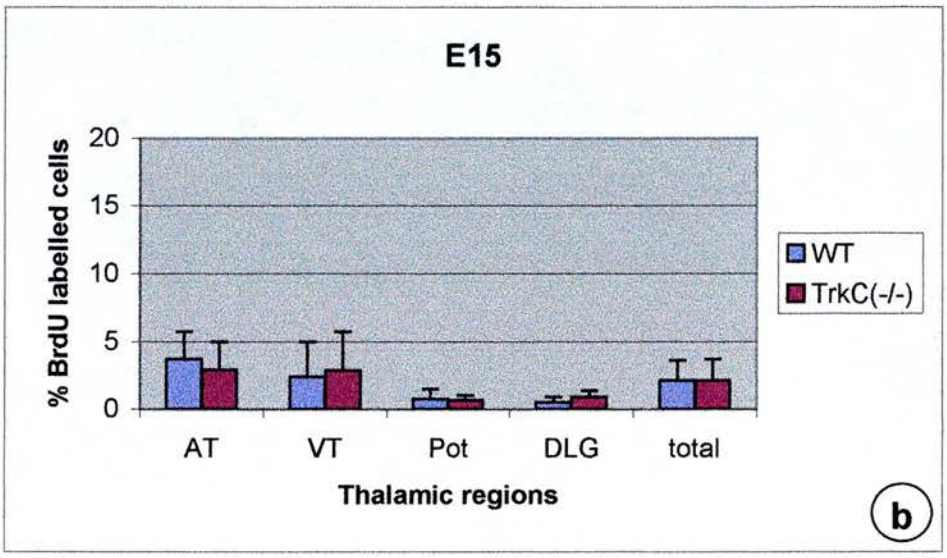
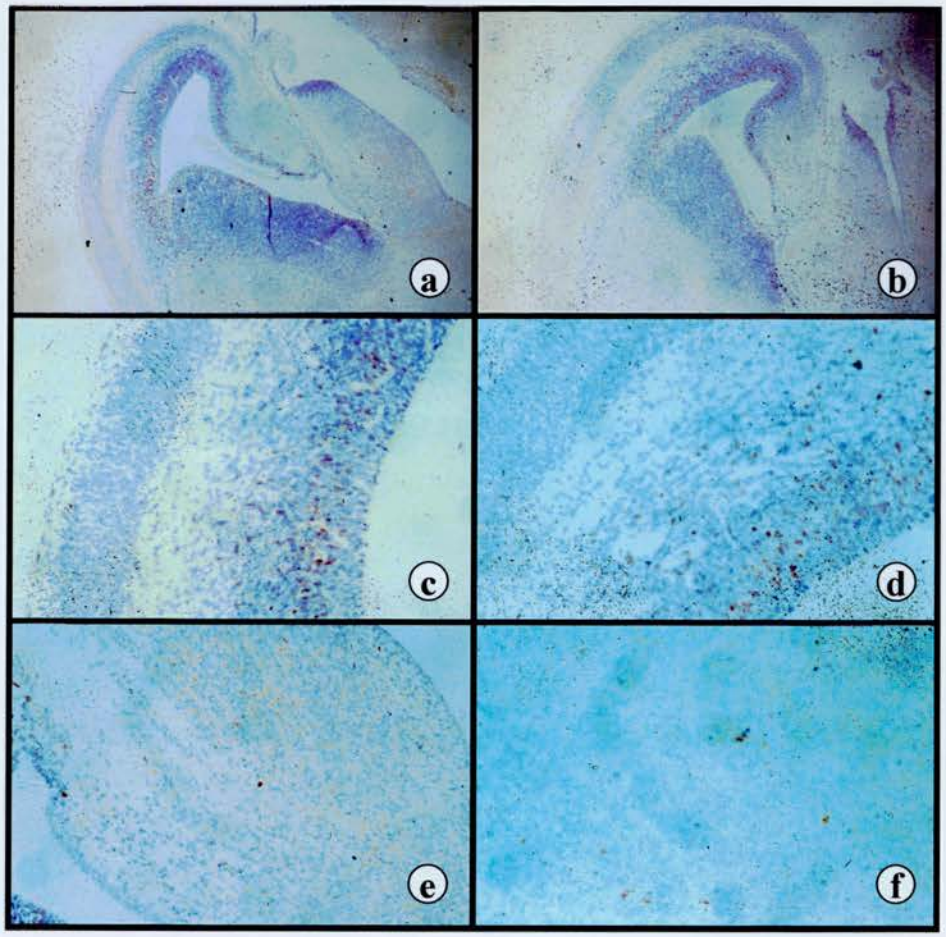

E15
% BrdU labelled cells
20
15
10
5
0
AT
VT
Pot
DLG
total
Thalamic regions
WT
TrkC(-/-)
b

Figure 7.4

Photomicrographs of E17 wild type and *trkC* knockout brains in anterior sections (section at 20% in anterior-posterior axis) in coronal plane. (a, b) Low magnification pictures of BrdU labeled cells (brown cells) of wild type and *trkC-/-* embryos. (c, d) Higher magnification of BrdU labeled cells in the cerebral cortex in wild type and mutant. (e, f) Higher magnification of BrdU labeled cells in the wild type and the mutant thalamus. Numbers of BrdU labeled cells in the thalamus at this age is very low.

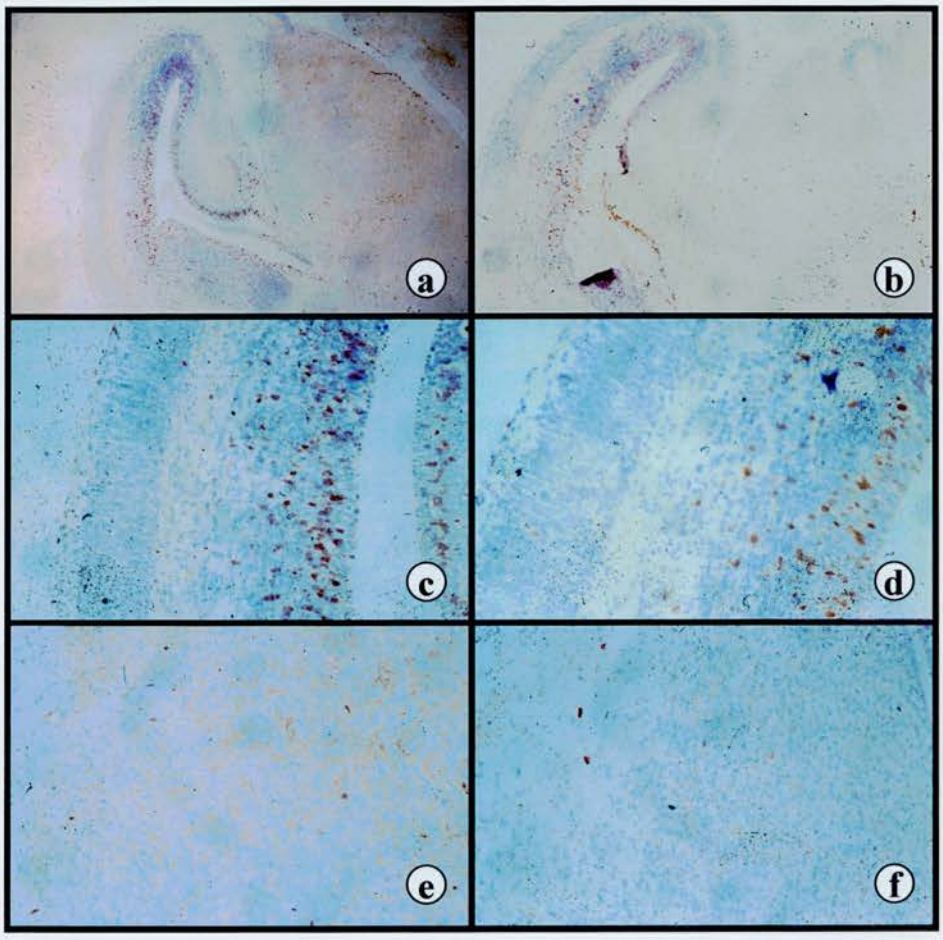


250μm (a, b)

100μm (c-f)

Figure 7.5

Photomicrographs of E17 wild type and *trkC* knockout brains in posterior sections (section at 80% in anterior-posterior axis) in coronal plane. (g, h) Low magnification pictures of BrdU labeled cells (brown cells) of wild type and *trkC-/-* embryos. (i, j) Higher magnification of BrdU labeled cells in the cerebral cortex in wild type and mutant. (k, l) Higher magnification of BrdU labeled cells in the wild type and the mutant thalamus. Note: Fewer labeled cells are found in E17 *trkC-/-* posterior cortex than in E17 wild type posterior cortex.

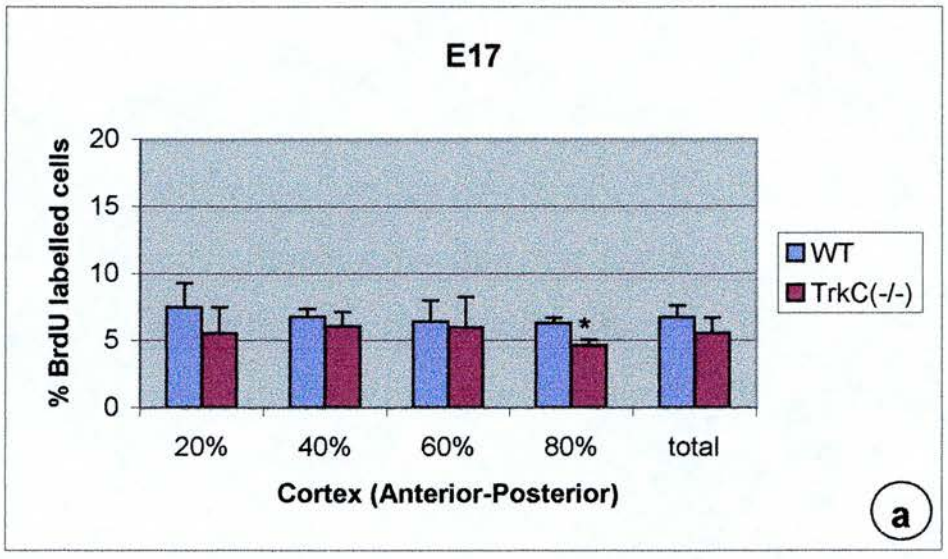


250μm (a, b)

100μm (c-f)

Figure 7.6

Graphs showing labeling index (percentage of BrdU labeled cells) in E17 cortex and thalamus of wild type and trkC knockout brains. (a) Shows BrdU labeling index in the E17 wild type and *trkC-/-* cerebral cortex. (b) Shows BrdU labeling index in the E17 wild type and *trkC-/-* thalamus. A small but significant decrease in the percentage of BrdU labeled cells is found at the posterior (80%) section of the mutant cortex. No significant difference in the labeling index is found in any of the nuclei of the thalamus.


E17
% BrdU labelled cells
20
15
10
5
0
*
WT
TrkC(-/-)
20%
40%
60%
80%
total
Cortex (Anterior-Posterior)
a

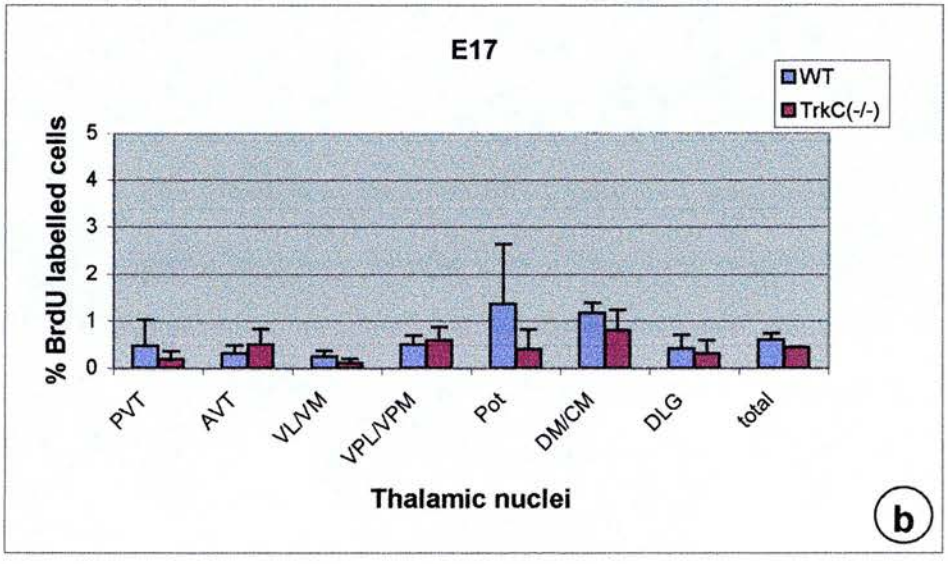
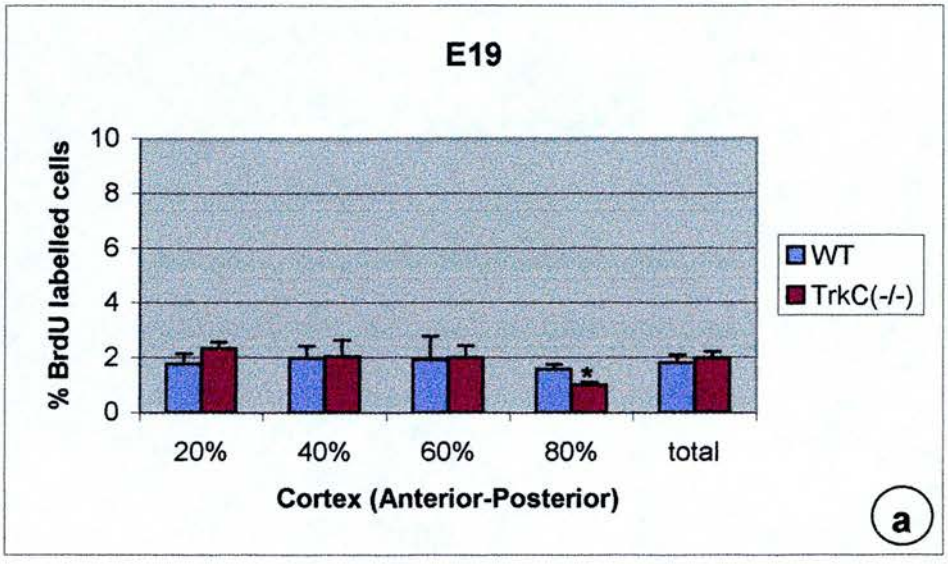

E17
WT
TrkC(-/-)
% BrdU labelled cells
5
4
3
2
1
0
PVT
AVT
VL/VM
VPL/VPM
Pot
DM/CM
DLG
total
Thalamic nuclei
b

Figure 7.7

Graphs showing labeling index (percentage of BrdU labeled cells) in E19 cortex and thalamus of wild type and trkC knockout brains. (a) Shows BrdU labeling index in the E19 wild type and *trkC-/-* cerebral cortex. (b) Shows BrdU labeling index in the E19 wild type and *trkC-/-* thalamus. A significant decrease in percentage of BrdU labeled cells is found at the posterior (80%) section of the mutant cortex. No significant differences in labeling index are found in the thalamus.


E19
% BrdU labelled cells
10
8
6
4
2
0
20%
40%
60%
80%
total
Cortex (Anterior-Posterior)
WT
TrkC(-/-)
*
a

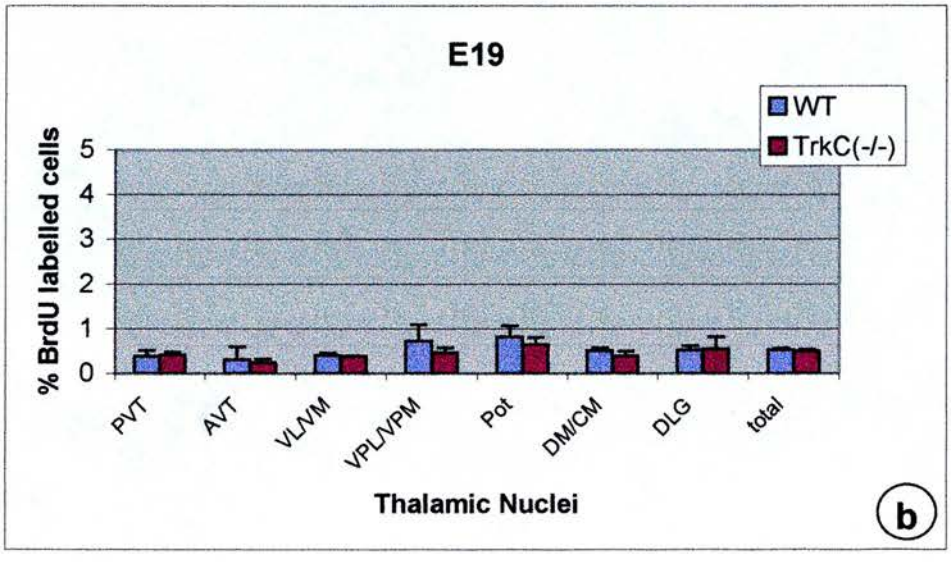
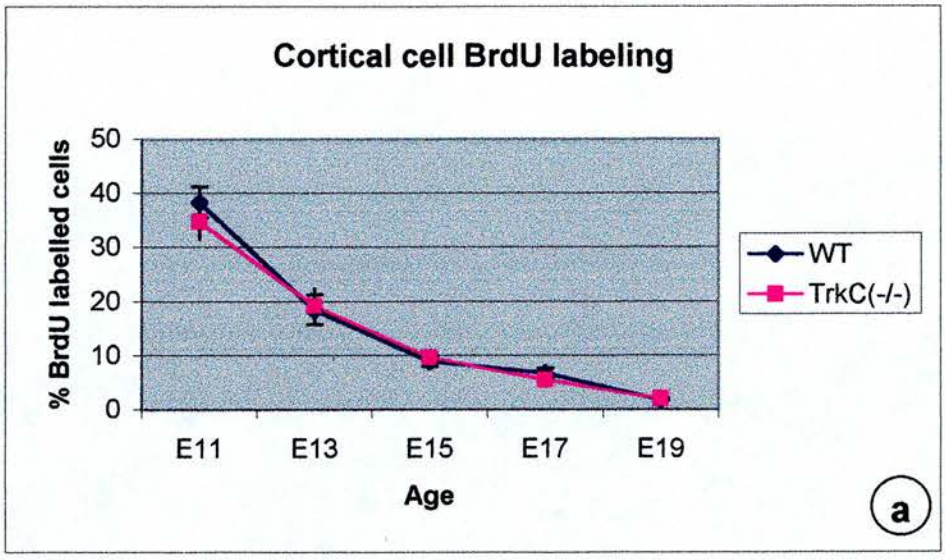
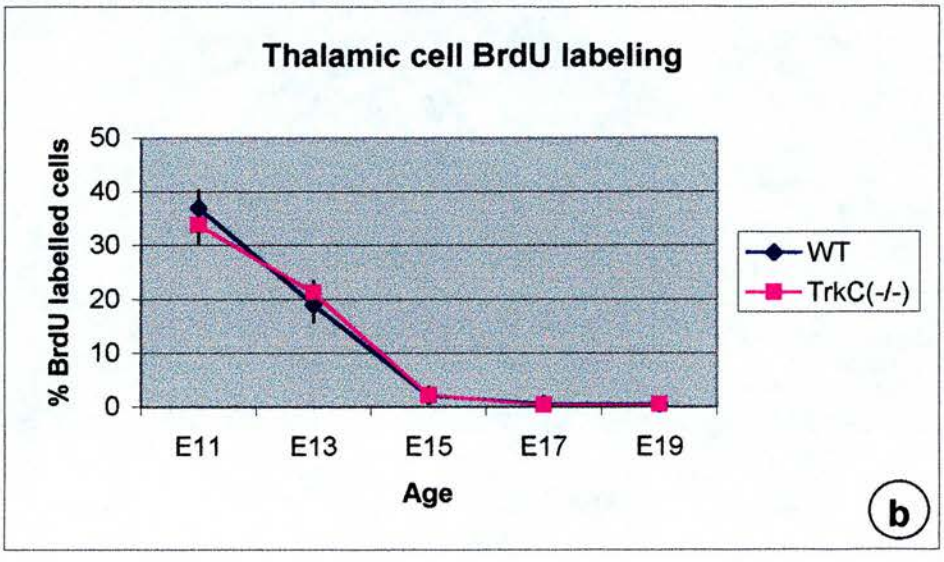

E19
% BrdU labelled cells
5
4
3
2
1
0
PVT
AVT
VL/VM
VPL/VPM
Pot
DM/CM
DLG
total
Thalamic Nuclei
WT
TrkC(-/-)
b

Figure 7.8

Summary graphs showing average labeling index (LI – percentage of BrdU labeled cells) in the cortex and thalamus of wild type and *trkC* knockout brains plotted against embryonic age. (a) BrdU labeling index in the wild type and *trkC-/-* cerebral cortex. Percentage of the labeled cells drops gradually and is lower than 10% after E15. Significant changes in LI of the mutant posterior cortex (Figure 7.5a and 7.6a) are insufficient to alter the overall LI. (b) BrdU labeling index in the wild type and *trkC-/-* thalamus is reduced earlier than in the cortex (below 5% after E15).


Cortical cell BrdU labeling
% BrdU labelled cells
50
40
30
20
10
0
E11
E13
E15
E17
E19
Age
WT
TrkC(-/-)
a


Thalamic cell BrdU labeling
% BrdU labelled cells
50
40
30
20
10
0
E11
E13
E15
E17
E19
Age
WT
TrkC(-/-)
b

Cerebral Cortex

At E11 and E13, when the cortex is thin and there is a fast proliferative rate, the BrdU positive cells were found throughout the thickness of the cortex, and the dense areas of immunopositive cells were often found in the middle of the cortex due to interkinetic movement of the cells and the occurrence of the S-phase cells at this central position (Figure 7.1a, c). This pattern of cell proliferation was the same as that found in the previous BrdU studies by Takahashi et al. (1991). As the cortical cells continue to proliferate, the E15 cortex becomes much thicker and many more cortical zones develop. The labeling index at this age was 8.89 $\pm$ 1.01 %. Highly immunopositive regions were found in subventricular zone and ventricular zone respectively (Figure 7.3). This continued to E19. But from E17, more BrdU positive cells were also found in the cortical plate. The LIs at E17 and E19 were 6.72 $\pm$ 0.85% and 1.81 $\pm$ 0.28 % (Figure 7.4 –7.6).

In the mutant cortex, the proliferative rates remained the same as those in the wild type until E17 (Figure 7.2, Figure 7.3). The average percentages of labeled cells from E11, E13, and E15 were 34.79 $\pm$ 3.23 %, 19.2 $\pm$ 3.12 %, and 9.58 $\pm$ 0.32 % respectively. Interestingly, I found a reduction in cell proliferation with some statistical differences ($p < 0.05$) in the sections 80% of the distance through the cortex at both E17 and E19. At E17, percentages of cells proliferating in this area (80% of the distance from the anterior section of the cortex) were 6.28 $\pm$ 0.41 % in wild type, and 4.66 $\pm$ 0.39% in *trkC-/-*. At E19, they were 1.58 $\pm$ 0.17 % in wild type, and 1.01 $\pm$ 0.1 % in the mutant. The area which was found to have a reduction in immunopositive cells corresponded to the occipital cortex, although the percentages of immunopositive cells for the cortex as a whole were the same.

Discussion

The motivation to carry out a study of cell proliferation in *trkC-/-* CNS followed the results in Chapter 3. I previously observed an unexpected, slight decrease in the percentage of cell death in early *trkC* mutant thalamus (E13), but this was not found in any other age studied. It was of particular interest because cells are highly proliferative at this age. I argued that if reduced cell death is found in this early stage without altered morphology or cell density in later ages of development, there might be other regulation to counterbalance the effects of the reduction in cell death. This regulation is likely to be cell proliferation. Both cell death and cell proliferation are essential parameters in regulating cell turnover and homeostasis (Kerr et al., 1980; 1994). Moreover, it has been suggested that cell death can occur not only in the postmitotic but also in the proliferating cells during embryogenesis (Blaschke et al., 1996; 1998). I thus postulated that *TrkC* signaling may be essential to homeostasis, in particular in the proliferation of early embryonic CNS cells, not only restricted to thalamic cells but also cells in other CNS regions such as cerebral cortex.

In this study, however, BrdU incorporation by most of the early thalamic and cortical cells aged between E11-E15 appeared to be normal. An effect was found in the later stage of development. Proliferative rates in a cortical area corresponding to occipital cortex (this area includes visual cortex) showed a significant reduction in the proportion BrdU labeled cells in the mutant. This reduction was found not only in one age but two (E17 and E19) indicating that *TrkC* signaling is required continuously for a certain period of time. In brief, *TrkC* may play a key role in cell proliferation and perhaps also in the decision to exit the cell cycle and differentiate at a specific period of development in specific brain areas (regional and age specificity) (Lewin and Barde, 1996; Segal and Greenberg, 1996; Kahn et al., 1999).

TrkC signaling plays a role in regulating proliferative rates in a restricted region in the brain

The roles of TrkC in brain development are still unclear although *trkC* mRNA is widely expressed and can be detected in the CNS early, before midgestation (Tessarollo et al., 1993). In their survival role, NT-3 and TrkC signaling can regulate cell death, often in early developmental stages, of many neuronal populations particularly in the PNS and among ganglionic neurons, but the effects in certain brain regions such as thalamus and cortex are subtle (see Chapter 3; Lotto et al., 1997; de la Rosa et al., 1994; ElShamy and Ernfors, 1996). Silos-Santiago et al. (1997) suggested that TrkC signaling might not play a major role in promoting survival in CNS development.

The role of TrkC in cell differentiation is also still controversial. Ghosh and Greenberg (1995) reported that NT-3 and TrkC signaling promote cortical neuronal differentiation while another study done by Silos-Santiago et al. (1998) reported that it does not play a major role in the differentiation of CNS neurons during embryonic development (Silos-Santiago et al., 1998).

Increasing evidence suggests that TrkC may play a role in the proliferation and differentiation of CNS cells (de la Rosa et al., 1994; Kumar et al., 1998; Engel and Wolswijk, 1996). But the effects are found at a relatively later stage of development and are thought to regulate gliogenesis rather than neurogenesis (see discussion below). In spinal cord, the lack of NT-3 or TrkC affected cell proliferation throughout the entire subventricular zone (Kahn et al., 1999). However, here I found that in *trkC-/-* mice, the proportions of proliferative cells were decreased only in a restricted region of the brain which corresponds to the occipital cortex, while there was no any other significant difference observed in the thalamus or other cortical areas. Within the cortical areas studied, the occipital cortex appeared to express the most *trkC* mRNA (see ISH result in Chapter 3). These findings indicated that TrkC signaling is regulates cell proliferation in a regional specific pattern in the CNS.

Although gross morphology appeared to be normal during embryonic development, both body size and brain size in the *trkC-/-* homozygous mice were reduced markedly in postnatal life. Thus it is possible that the reduction in proliferation at the later stage of development (peri- and post- natal) may contribute to this abnormality. Since most homozygous mutant die in early postnatal life, it is also possible that this late stage of cell proliferation may have essential physiological functions for foetal development (Klein et al., 1994).

Possible role of NT-3 and TrkC signaling in visual cortex

The neurotrophins have been implicated in shaping and remodeling the connectivity of neural circuits (Okuno et al., 1999). Both neurotrophins and their receptors are localised in numerous visual centers from the retina to the visual cortex (see review by Von Bartheld, 1998). BDNF and TrkB receptors are the most studied molecules in the development of retina and visual pathways. For example, BDNF can prevent the death of cultured retinal ganglion cells in many animal species (see Lewin and Barde, 1996). BDNF (and also NT-3) can prevent naturally occurring cell death in the isthmo-optic nucleus (Von Bartheld et al., 1994). In visual cortex, BDNF and NT-4/5 can influence the patterning of projections and the formation of ocular dominance columns (Cabelli et al., 1995). Moreover BDNF regulates dendritic growth and modulates plasticity in developing visual cortex (McAllister et al., 1995; Ghosh, 1996). In contrast, much fewer studies have been carried out to investigate the role of NT-3 in the development of this area. Yet McAllister et al. (1997) demonstrated that NT-3 opposes BDNF action on dendritic growth in visual cortex. In addition, NT-3 is known to regulate proliferation and survival of glial cells in the optic pathways (von Bartheld, 1998).

In the *in situ* hybridisation studies, *NT-3* mRNA is found to be transiently expressed in the neonatal cortex and thalamus and declines during the first two postnatal weeks, while levels of *BDNF* and *NGF* mRNAs expression increase during postnatal development and reach mature levels around 3 weeks of age (Schoups et al., 1995; Ringstedt et al., 1993). As for the family of *trk* receptors, *trkA* is expressed at very low levels in most CNS areas while *trkB* and *trkC* are expressed both in the

developing LGN and the occipital cortex, mostly in overlapping patterns (see Chapter 3; Ringstedt et al., 1993). In addition, *trkC* is widely expressed in other brain regions and in non-neuronal tissues where *trkA* or *trkB* are excluded (Tessarollo et al., 1993). In the occipital cortex, *trkC* is found to be relatively more highly expressed than in other rostral neocortical areas (i.e. there is a rostro-caudal gradient of *trkC* expression, see Chapter 3).

In summary, the findings of strong *trkC* expression in visual cortex and the proliferative defects found in *trkC* mutants indicated that TrkC and NT-3 are as important as TrkB and BDNF in playing a role in the development and maturation of the visual cortex.

Possible role of NT-3 and TrkC signaling in gliogenesis

The reduction in the number of BrdU labeled cells in the *trkC* mutant brain could be because the lack in TrkC signaling 1) shortens S-phase and increases cells exiting the cell cycle and differentiating (and thus make us see fewer labeled cells) or 2) reduces overall proliferative rates. So far, there is still no clear evidence supporting the first possibility while many have reported that impaired NT-3 and TrkC signaling results in a reduction of cell proliferation in many populations (Das et al., 2000; Kahn et al., 1999; Kumar and de Vellis, 1996).

It is not known whether the mutant cells that exhibit the proliferative defect are undifferentiated cells, neurons or glia. This requires further investigation but there is a strong implication that proper development of glial populations (gliogenesis) requires TrkC signaling. I found that the reduction in the percentages of BrdU labeled cells in occipital cortex occurred late, around the time of extensive gliogenesis in the cortex, when neurogenesis is almost complete (Levers, 1999; Edgar, 1998). Moreover, TrkC receptor protein is found in several types of glioma cells (oligodendroglia - OL, CG-4, and C6) (Kumar and de Vellis, 1996). In P2 mice lacking NT-3 or TrkC, the numbers of glial cells of each type, namely O-2A progenitor cells, oligodendrocytes (OL), astrocytes, and microglial cells in the spinal cord are markedly reduced (Kumar et al., 1998; Kahn et al., 1999). Previous studies

also showed *in vitro* that NT-3 (along with platelet-derived growth factor type AA, PDGF-AA) promoted proliferation of O-2A progenitor cells in both perinatal optic nerve and adult spinal cord (Barres et al., 1994; Engel and Wolswijk, 1996). Kumar et al. (1998) also suggested that the most likely mechanism of proliferative action of TrkC signaling in these glial population is via the Ras and MAPK pathway.

Chapter 8: General Conclusion

8.1 Summary of main points

In this thesis, my main interest was to investigate the process of programmed cell death (PCD) and the mechanisms and factors regulating it in the developing thalamus at the tissue level. I have used both *in vivo* and *in vitro* approaches to look at this process in the embryonic and early postnatal mice.

Measuring thalamic PCD

At the beginning of the thesis, I successfully identified one of the most common and prominent modes of PCD in the thalamus, apoptosis, both *in vitro* and *in vivo*. I found that both nuclear morphology revealed by DNA fluorochromes and TUNEL staining confirmed the characteristics of apoptotic cells. *In vitro*, dying embryonic thalamic cells are mostly apoptotic. I also confirmed that PCD actually occurred in this tissue *in vivo* by observing PCD with TUNEL studies.

Occurrence of thalamic PCD

I conducted a study identifying the time course of PCD *in vivo*. Cell death is found throughout the late gestation and early postnatal stages of thalamic development, and the results correspond with previous reports in other developing tissues. The work done by Glucksmann (1951) and Hensey and Gautier (1998) showed that cell death occurs as early as the blastocyst stage, during gastrulation and extended to the period of neurogenesis. However, the PCD occurring at these stages is rare and random. Similar results were found in my studies of PCD in the early thalamus at E13. It is thought that cell death at this stage does not involve trophic support as a survival-determining factor (Hensey and Gautier, 1998), but is likely to be regulated by other as yet poorly understood mechanisms. On the other hand, thalamic PCD was found extensively in later stages of the development (i.e. during

the perinatal period, postnatal day 1). In addition, cell death at this later stage is patterned and distributed differently in different thalamic nuclei (with the highest PCD found in VPM). This uneven PCD distribution emphasizes the heterogeneity among thalamic nuclei e.g. the time or extent in establishment of synaptic organisation and axonal pathways of each thalamic nucleus might be different and result in different PCD distributions.

In tissue culture, the increase in cell death in thalamic cells is found earlier than *in vivo* (*in vitro* day E18), although this is still around the period of early cortical innervation. Survival of these cultured thalamic neurons is increasingly dependent on several more factors as the cells mature. For example, the cells require a high cell density environment (Chapter 2), trophic supply from medium conditioned with neuronal tissues (Chapter 3 and 4) and perhaps thalamic activity involving K^+ depolarisation to maintain survival (Chapter 4 and 5). However, the last possibility is controversial since K^+ depolarisation can only promote thalamic survival in explants but not in dissociated cells.

The different period at which there is increased cell death *in vitro* (E18) and *in vivo* (P1) suggests that there might be a window (E18-P1) during which thalamic cells need to adapt or switch from the stage of intrinsic self-sufficient viability to the stage of dependency on extrinsic factors (i.e. the target). This transition period is interestingly matched with the time thalamic cells extend their axons to innervate the target (Rennie, 1994; Lotto et al., 1997). Similarly, in *Xenopus* embryonic cells, there is also such a transition period before the period of normal *in vivo* PCD. During this period, cells are sensitive to external apoptotic stimuli but give a partial and delayed apoptotic response (Hensey and Gautier, 1997).

Matching the Neurotrophic hypothesis with PCD in thalamus

A very plausible mechanism to explain PCD in the thalamus is the Neurotrophic Hypothesis. This was originally proposed in the peripheral nervous system but the application in the CNS is poorly understood (see review by Oppenheim, 1999). The concept of the hypothesis is that neurons are initially

overproduced and PCD occurs to neurons that fail to compete for limiting amounts of target-derived trophic molecules during the innervation period. In my study, I tested two major possibilities, which could fit with this hypothesis. Firstly, I showed the importance of neurotrophic factors (neurotrophins and unidentified trophic factors from medium conditioned with neuronal tissues) on thalamic survival both *in vivo* and *in vitro* (Chapter 3 and 4). And later, the experiments in *Pax-6* mutant cells also indirectly indicated the significance of the thalamocortical innervation (Chapter 6). Although there is no definite method to prove this hypothesis, the findings in my investigation strongly support this applicability of the hypothesis in the CNS.

Regulation of thalamic PCD

1. Possible roles of neurotrophins in the thalamus

Prime candidates for molecules that are likely to control thalamic cell survival are neurotrophins. In the periphery, the survival and differentiation of particular neurons generally depends on individual neurotrophins (reviewed by Snider, 1994; Lindsay et al., 1994). The roles of neurotrophins in central nervous system development are likely to be more complex, as central neurons commonly respond to several neurotrophic factors, and their survival and differentiation *in vitro* requires multiple neurotrophic factors (Snider, 1994; Meyer-Franke et al., 1995). Multiple neurotrophins provide redundant growth and differentiation signals, but a more interesting possibility is that developing neurons use multiple neurotrophin signals to direct distinct aspects of neuronal growth and differentiation (McAllister et al., 1997).

In the collaborative studies with Dr R Beau Lotto in Chapter 3, we examined the survival-promoting role of neurotrophins and their receptors in the developing thalamus. We found that BDNF and its receptor, TrkB, but not other neurotrophins or their companion receptors, are essential for the survival of some thalamic populations. The effects from the manipulation of BDNF or TrkB are prominent around the period of target innervation (from around E17 both *in vitro* and *in vivo*) and before the time of the PCD peak. However, we cannot conclude that BDNF is

the only factor essential for thalamic survival in that study since BDNF alone is not sufficient for thalamic survival *in vitro*. This leads to the interpretation that the regulation of PCD in the CNS is likely to be more complex and may involve several trophic factors or mechanisms.

Another possible role of neurotrophins in thalamic and cortical development was also investigated in this thesis. In Chapter 7, I studied the proliferative defect in TrkC knockout mice and found that TrkC might be involved in cell proliferation particularly in the later stage of cortical development, which possibly involves gliogenesis. Thus neurotrophins may also have other roles than controlling survival in the developing forebrain.

2. Dynamic roles of intrinsic- and target-derived trophic factors

My work and other previous investigations suggested that there are likely to be more than one trophic factor supporting thalamic viability and that conditioned medium can promote survival of the majority of thalamic cells *in vitro*. However, the purification of trophic factors from this medium has been unsuccessful. In order to gain a better understanding of the nature of this medium while the factors within it are unknown, I tested the specificity of media pre-conditioned with various sources of tissue including thalamus and cortex to promote thalamic survival. Surprisingly, not only cortical conditioned medium but also conditioned medium of other neuronal tissues could promote thalamic survival. This means that trophic factors required by thalamus are present in many neuronal tissues including the thalamus itself. Dissociated thalamic neurons are able to survive in E19 thalamic conditioned medium providing that the activity of the conditioned tissue is maintained by K^+. This suggests that thalamic cells do not completely switch their trophic responsiveness from the intrinsically produced trophic factors to target-derived factors during cortical innervation period, while such a switch has been reported to occur in several other neuronal systems (Davies, 1996). In the case of the thalamus, it is thus possible that the reduction in the level of intrinsic trophic production (rather than the switch in trophic responsiveness) crucially regulates the viability of thalamic

neurons during the innervation period and later forces the thalamic cells to change their source of trophic supply and become dependent on the target cerebral cortex.

It is not clear what mechanisms make the thalamus reduce its own production of the growth factors at E19. In my opinion, it may be due to both an intrinsic clock and the remodelling for establishment of thalamocortical circuits. The intrinsic mechanism signaling thalamic cells to become less viable is one of the aspects that is worth further investigation.

3. Possible roles of neuronal activity in regulating thalamic survival and possible interaction of K^+ and neurotrophin signaling

As discussed above, there may be more than one factor that regulates thalamic survival. One possibility is the activity-mediated regulation of neuronal survival (Ghosh et al., 1996). Magowan and Price (1996) have shown that K^+ depolarisation promotes growth and survival of thalamic neurons during the period of innervation before birth (the peak of *in vivo* PCD). I found a similar effect in this study. I also further investigated whether thalamic trophic effects of K^+ would be compromised in the mice lacking one of the neurotrophin receptors (either TrkB or TrkC). No significant difference in cell death was found among the wild type and the mutants in response to treatment with K^+. Thus, I found no direct correlation between the effect of K^+ activity and neurotrophin receptor signaling. Therefore, it leaves the possibility that activity-dependent survival promoting effects mediated by K^+ may use other neurotrophin-independent mechanisms to regulate thalamic PCD.

4. Regulation of PCD during the innervation period: possible essential role of the innervating axons

I investigated the possible role of a developmental transcription factor, Pax-6, in thalamic survival. Although expressed in both diencephalon and telencephalon, this gene is not detectable in the developing dorsal thalamus at later stages of development. Striking observations were found in this study: 1) an increased cell death was found only in the thalamus (but not in the cortex) at the time before birth

(corresponding to the innervation period), while 2) thalamic cells cultured up to this age *in vivo* (E19-P0 DIV) survive normally when supplied with sufficient trophic support, and 3) the cortex-derived factors were not absent in the mutant. This suggests that the death of thalamic cells is not due to an intrinsic defect or a reduction in trophic production from the target but is likely a result of the disruption of thalamocortical axons found in the mutant during the innervation period. This finding supports the concept of the significance of innervating axons in controlling cell survival.

In final summary, there are several components controlling survival and PCD in developing thalamus particularly during the critical innervation period (E17 to the early postnatal period in mice). First is the intrinsic properties of the thalamus i.e. a) internal clock, b) thalamic activity, and c) regulation of intrinsic trophic production. Secondly, survival in the developing thalamus could be regulated by external support: a) neurotrophic factors supplied by the target, and b) proper axonal growth, guidance and establishment of connection.

8.2 Future works

There are three main areas where I would like to extend my research in the future to gain a better understanding of thalamic PCD related with thalamic cell growth.

1) An investigation of the intrinsic properties of the developing thalamus: the *in vitro* investigation of Bcl-2

I would like to investigate cellular and molecular mechanisms regulating thalamic PCD e.g. to study whether regulation of the thalamic PCD involves metabolic activity, protein synthesis, transcription regulation, and/or is controlled by apoptotic proteins. Bcl-2, as well as other cell death regulating elements (both proteins and genes), plays a key role in the intrinsic (intracellular) signaling of apoptosis. Recent studies have shown that it also regulates neuronal growth and

regeneration in CNS and PNS. Moreover, exogenous Bcl-2 has been successfully introduced to the cells *in vitro* by using Cre/Lox-P system (Sato et al., 1998). With this technique, I can investigate the possible role of this protein during the critical period by introducing and expressing the gene in thalamic cells cultured at different ages. Studies of the distribution of this protein in the brain and investigations of the defects in the thalamus of mice lacking or overexpressing Bcl-2 could also be meaningful.

2) An investigation of other possible trophic factors and guidance molecules: a possible role of extracellular matrix molecules

It is interesting that there is no collagen but there is a high proportion of other glycosaminoglycans in the extracellular matrix of neuronal tissue. Many of these molecules are known to promote the growth of thalamic neurons as well as other neuronal populations (Emerling and Lander, 1996; Nissen et al., 1999). But the function of these molecules in survival has not been established. I wish also to study the role of molecules such as the family of chondroitin sulfates in thalamic development, using both *in vivo* and *in vitro* approaches.

3) An investigation of the interaction between thalamic neurons and cortical neurons *in vitro*

Embryonic stem (ES) cells and transgenic mice ubiquitously expressing a tau-GFP (*g*reen *f*luorescent *p*rotein) fusion protein have recently been generated in our laboratory (Pratt et al., 1999). This is a useful animal model to provide a universal source of labeled cells for cell mixing experiment *in vitro*. For example, with a labeling of one type of cell, I hope to be able to study cell interaction and behavior between thalamic neurons and other cell types including the target cortical neurons. I may observe the formation of synapses or neuronal connections, which may strengthen the ability of thalamic neurons to survive in culture.

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Appendix

CULTURE MEDIUM

	Sigma Cat No.(Final conc.)
Mix together in a sterile beaker:	
100ml F12 (Hams)*	N4888
100ml Delbeco's modified Eagles' medium (DMEM)	D5671
1mg insulin	I6634 (5μg/ml)
2mg apo-transferrin**	T1147 (10μg/ml)
3ml HEPES buffer	H0887
0.24g Na_2HCO_3	S5761 (0.12g/ml)
3ml antibiotics (Gentamycin + Kanamycin)***	G1264 + K1377
2ml putrescene****	P5780 (16.11μg/ml)
20μl progesterone****	P8783 (6.29ng/ml)
20μl Na_2SeO_3****	S5261 (5.2ng/ml)
2ml $_L$-glutamine****	G2128 (25μg/ml)

Notes:

* **F12** contains more nutrients than DMEM (including amino acids and vitamins)

** **Transferrin** is a beta glycoprotein (iron transport protein in the blood). It transports iron to (and possibly within the cell) in culture. It may also have a detoxifying role).

*** **Antibiotics:** **Gentamycin sulphate** (100mg)
Kanamycin sulphate (200mg)

Make up to 20ml with double distilled water (sterile), then filter sterilise. Store in 1ml aliquots at –20°C.

**** **Supplements:**

1) 100μM **putrescene** (161.1mg/100ml in sterile double distilled water, filter sterilise. Store in 4ml aliqouts at –70°C.

Putrecene is the decarboxylated product of the amino acid ornithine. It is needed as a precursor for the synthesis of polyamines which are involved in cell growth and proliferation.

2) 20μM **progesterone** (6.29mg/100ml in ethanol). Store in 1ml and then 50μl aliquots at –70°C

3) 30μM **Na_2SeO_3** (5.2mg/100ml in sterile double distilled water, filter sterilise). Store in 1ml and then 50μl aliqouts at –70°C.

Selenium is a co-factor of glutathione peroxidase which is located in the cytosol and catalyses the reduction of radicals by the antioxidant glutathione.

4) 0.2M **$_L$-glutamine** (6.344g/100ml in sterile double distilled water, filter sterilise). Store in 2ml aliqouts at 50μl at –70°C.

$_L$-glutamine is a non-essential amino acid which is an important precursor for nucleotide and structural protein synthesis. It is required of $_L$-glutamate and GABA synthesis. At high concentrations is may have neurotoxic side effects.